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THE ACTIVATION OF CHYMOTRYPSINOGEN-B

by

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A THESIS

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE ACTIVATION OF CHYMOTRYPSINOGEN-B", submitted by C. Owen Parkes in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Date

July 12, 1965.

ABSTRACT

Recent work has shown chymotrypsinogen-A and chymotrypsinogen-B to be structurally similar and the enzymes derived from them to possess comparable specificities towards bonds in the polypeptide glucagon. Further details of the enzymic properties of chymotrypsin-B have been examined, and the tryptic activation of chymotrypsinogen-B has been studied. The rate constants for the hydrolysis of N-acetyl-L-tyrosine ethyl ester and the K_i 's for three competitive inhibitors were all found to be similar with chymotrypsin-B and chymotrypsin-A₄. Chymotrypsin-B could be acylated with the reagents cinnamoylimidazole, trimethylacetylimidazole, and anisoylimidazole, but the deacylation rates of the acyl-chymotrypsin-B's were approximately five times faster than those of the corresponding acyl-chymotrypsin-A₄'s.

Under rapid activation conditions, chymotrypsin-B was generated faster than chymotrypsin-A, but the maximum esterase activity was lower. A specific activity towards N-acetyl-L-tyrosine of 3.3 resulted from activation of chymotrypsinogen-B for 5 minutes at 0°, while chymotrypsinogen-A activation required 30 minutes to produce an activity of 4.6 at the same temperature. Calcium ions and the chymotryptic inhibitor, indole, had no effect upon the activation rate, but were observed to stabilize the active enzyme and prevent loss of esterase activity.

Although the tryptic hydrolysis of an arginine-isoleucine bond, 15 residues from the N-terminus of the molecule, has been shown to be the first step in the activation of both zymogens, the present work has indicated differences in the subsequent autolytic cleavages. No activation dipeptide was found to be liberated during rapid activation of chymotrypsinogen-B. Instead, autolysis caused at least two new breaks in the molecule near residue 140. Only after slow activation were these bonds extensively cleaved, with the probable loss of a peptide to produce an enzyme molecule with a specific esterase activity of 2.0. Isolation of the

S-carboxymethyl chains of chymotrypsin-B on a column of DEAE-cellulose and end group analysis indicated that one of the bonds broken may be between leucine and lysine and the other appeared to involve the carboxyl group of a tyrosine residue. Thus the final products from slow activation of both zymogens are enzyme molecules composed of three polypeptide chains held together with disulfide bonds.

It has been suggested that activation of chymotrypsinogen results in the rearrangement of the molecule to bring the components of the active site into the correct spatial relationship necessary for enzymic activity. Ultraviolet difference spectrophotometry showed the activation of chymotrypsinogen-B resulted in a spectral change very like that observed during the activation of chymotrypsinogen-A, while optical rotatory dispersion measurements suggested a change in tertiary structure without alteration of the helical content of the molecule. Ultracentrifugal examination of chymotrypsin-B indicated that the enzyme had a tendency to dimerize at pH 3.0. The extent of dimerization, as judged by a comparison of $S_{20,w}$ values, was less for chymotrypsin-B than for chymotrypsin-A₄. Free boundary electrophoresis of the zymogen and active enzyme showed that the isoelectric points of both proteins were very similar. Chymotrypsin-B had an isoelectric point of 5.03 while that of chymotrypsinogen-B was 5.2.

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List of Abbreviations

A-CHT-B	anisoyl-chymotrypsin-B
A-I	anisoyl imidazole
ATEE	N-acetyl-L-tyrosine ethyl ester
C-CHT-A, C-CHT-B	cinnamoyl-chymotrypsin-A, -B
CHT-A, CHT-B	chymotrypsin-A, -B
CHTG-A, CHTG-B	chymotrypsinogen-A, -B
C-I	cinnamoylimidazole
CM-cellulose	carboxymethyl-cellulose
CP-A	carboxypeptidase-A
DEAE-cellulose	diethylaminoethyl-cellulose
DFP	diisopropylfluorophosphate
DPC	diphenylcarbamyl chloride
$E_{1\%}^{1\text{cm}}$	absorbance of a 1% solution in 1 cm. light path
ϵ	molar extinction coefficient
K_m	Michaelis constant
NTMA	p-nitrophenyltrimethyl acetate
STI	soybean trypsin inhibitor
TMA-I	trimethylacetylimidazole
TMA-CHT-B	trimethylacetylimidazole chymotrypsin-B
TPCK	N-tosylphenylalanine chloromethyl ketone
Tris	tris(hydroxymethyl) amino methane

I. INTRODUCTION

Although chymotrypsinogen-A* (CHTG-A) was crystallized from bovine pancreas by Northrop and Kunitz (1) in 1930, it was not until 1946 that Laskowski (2) found in the same gland a new protein called "protein B" that was eventually shown to possess potential proteolytic activity. This zymogen, originally thought to be a deoxyribonuclease (2), could be activated by trypsin and enterokinase, and was proposed to be related to chymotrypsinogen-A (3). The work of Fruton (4) outlined the specificity of the active enzyme, showing it to be similar to chymotrypsin-A₄ (CHT-A₄), while Brown et al. (5) crystallized the enzyme, calling it chymotrypsin-B (CHT-B) and the zymogen, chymotrypsinogen-B (CHTG-B). Possibly because of its fairly recent isolation and the low yields of purified protein, CHTG-B was not considered to be a major component of pancreatic juice (10). However, Keller, Cohen and Neurath (6) have shown that CHTG-B and CHTG-A each form 16% of the total protein in bovine pancreatic juice, so that the B chymotrypsin system may well be of considerable physiological importance.

The isolation and purification of CHTG-B was first performed by Laskowski and co-workers (2,3) from a sulfuric acid extract of beef pancreas. Ammonium sulfate fractionation of

* The terminology proposed by Rovery and Desnuelle (10) will be followed throughout this work.

this extract was followed by crystallization from acetate buffer, pH 5.5. Column chromatography on carboxymethyl cellulose (CM-cellulose) (7) showed the presence of significant amounts of "neochymotrypsinogens" (8); the method was therefore modified by the inclusion of diisopropylfluorophosphate (DFP) in all solutions above pH 4.0 (9). The level of C-terminal tyrosine was thereby reduced to 5-6% and the free chymotryptic activity to 0.2%.

In 1960 Röver et al. (11) used a similar acid extraction and ammonium sulfate fractionation in the initial steps, but final purification of the zymogen was achieved on CM-cellulose columns, using stepwise elution with citrate buffers. A small inactive peak was eluted before the main bulk of CHTG-B by 0.05 M buffer, pH 4.6. Although the protein was apparently homogeneous as judged by re-chromatography on diethylaminoethyl cellulose (DEAE-cellulose), the potential k' against N-acetyl-L-tyrosine ethyl ester (ATEE) was only 2.6* (11).

The product from Laskowski's laboratory has been more extensively studied and was demonstrated to be homogeneous in the ultracentrifuge (13) and by free boundary electrophoresis, but a small amount of impurity was evident from solubility experiments (12). The amount of neochymotrypsinogens as judged by C-terminal analysis may vary widely from one preparation to another (16,28). Originally, the potential activities

* k' = meq of substrate hydrolysed per minute per mg. enzyme nitrogen. Maximum theoretical value for CHT-B = 3.36.

reported by Laskowski were based on the casein method of Kunitz (14), the purity of a particular specimen being judged with reference to the average value obtained from a large number of samples. This method is not directly comparable to the esterase method because variations from one sample of casein to another may occur, as pointed out by Enenkel and Smillie (15). In 1964 Krehbeil et al. (16) reported activities using ATEE as substrate which indicated a k' of approximately 3.0 for this material. Assays carried out in this laboratory on a sample kindly donated by Dr. Laskowski gave a potential k' of 2.86.

In 1960 work was begun in this laboratory on the purification of CHTG-B, and a modified chromatographic method developed (15). Initial sulfuric acid extraction was employed, followed by fractionation between 0.2 and 0.4 saturated ammonium sulfate. Refractionation between 0.3 and 0.4 saturated ammonium sulfate at pH 3 gave a product of potential $k' = 1.5$. This value rose to 3.0-3.2 after chromatography on a column of CM-cellulose (0.6 meq per g capacity) in sodium citrate buffer, pH 4.47, with a linear concentration gradient from 0.03 M to 0.15 M. The free activity was 0.01-0.03% and assay with N-trans-cinnamoylimidazole (17) indicated that the preparations were 90-95% activatable.

The molecules of CHTG-A and CHTG-B are similar with respect to amino acid composition, being peculiar in having a low content of histidine, methionine, arginine and tyrosine; while measurements of the hydrodynamic properties have indicated a marked resemblance in shape. A chymotrypsinogen from

Table I
Some Properties of Chymotrypsinogen-A
and Chymotrypsinogen-B

Property	CHTG-A	CHTG-B
Molecular weight	25,100(22) *	24,500(23)
Isoelectric point	9.5(24), 9.1(12)	5.2(23), 5.2(10)
$S_{20,w}^O$	2.58 S (22)	2.6 S (23)
Number of residues	246(18)	235(26)
Disulfide bonds	5(18)	5(26)
Arginine and lysine residues	18(18)	16(26)
Glutamic and aspartic residues	35(18)	39(26)
Amide groups	23(18)	16(9)
Methionine	2(18)	4(9,26)
Tyrosine	4(18)	3(9,26)
$E_{1\%}^{1\text{cm}}$	20.0 at 282m μ (29)	18.7 at 280 m μ (23)

*

Figures in parentheses indicate references.

porcine pancreas (21) shows comparable properties. Some of the points of similarity and difference are listed in Table I. Clearly the most significant structural difference is in the number of free side chain carboxyl groups and basic groups. This, in turn, probably accounts for the chief physical difference, that of isoelectric points. Mainly as a result of the work of Hartley (18), and Keil and Sörm (19), the amino acid sequence of CHTG-A is known, but knowledge of the sequence of CHTG-B is, as yet, fragmentary. Nevertheless, the sequences that have been elucidated in CHTG-B bear a remarkable resemblance to the sequence of CHTG-A in those areas which are thought to be functional in the enzyme (around serine-195, histidine-40 and histidine-57). The full sequence of CHTG-A, as described by Hartley, is given in Figure 20 together with the sequences of the partially ordered disulfide and tryptic digest peptides from CHTG-B available at the time of writing.

The enzymic activity of the most readily available and stable form of CHT-A, CHT-A₄, has been studied in great detail from a number of angles. Much of this work has been aimed at elucidating the details of the "active" site and the functional role of the serine and histidine residues involved in catalysis. The present picture is summarized in two recent papers by Bender et al. (30,31). Evidence for the involvement of serine was given by Jansen et al. (32) who showed that incubation with DFP inhibited CHT-A₄, and that 1 mole of phosphate was incorporated per mole of enzyme (33). Shortly afterwards, Schaffer et al. (34) isolated O-phosphoryl serine. There was a good deal of circumstantial evidence implicating

histidine in the active site of CHT-A₄ (35) but it was left to Schoellman and Shaw (36), using the reagent N-tosylphenylalanine chloromethyl ketone (TPCK) to demonstrate this conclusively. Reports on the enzymic properties of CHTG-B, on the other hand, are few and deal mainly with its specificity.

In 1947 Keith et al. (3) showed that soybean trypsin inhibitor (STI) acted in the same way on both CHT-B and CHT-A₄, while Ambrose and Laskowski (37) found that CHT-B digested casein, denatured egg albumin, denatured hemoglobin, edestin and CHTG-A more slowly than did CHT-A₄. CHT-B is also able to hydrolyse synthetic amide and ester substrates. Fruton (4) used a range of synthetic substrates to support the view that the newly isolated protein-B was in fact a chymotryptic zymogen; Desnuelle and Rivery (10) report that CHT-A can be distinguished from CHT-B by the rate of hydrolysis of ATEE in the presence and absence of 30% methanol. Methanol has no effect upon the activity of CHT-A towards this substrate, while it strongly depresses the hydrolysis by CHT-B.

The recent report by Enenkel and Smillie (38) of the hydrolysis of glucagon and synthetic ester substrates clearly shows the similarity of the CHT-B and CHT-A₄ activities. In its endopeptidase action on glucagon, CHT-B shows a slightly lower specificity, as partial cleavage occurs on the carboxyl side of two leucine residues, but both A and B enzymes, when tested with benzoyl leucine ethyl ester, show some small activity. While many aspects of the catalytic activity of CHT-B remain uninvestigated, the enzyme has been used for sequence studies of pancreatic basic trypsin inhibitor by

Kassell et al. (20). These workers confirm the observations of slightly lower specificity for the B enzyme as compared to CHT-A₄.

Both CHTG-A and CHTG-B require treatment with trypsin, enterokinase or a similar enzyme before they exhibit chymotryptic activity. Generally, activation by trypsin has been used with both zymogens, although there are a number of recent reports (29,39) on the activation of CHTG-A by bacterial proteases. There are two methods commonly used to activate CHTG-A. "Fast" activation was first described by Jacobsen (40), and entails using high trypsin concentrations (about 40-50 moles CHTG-A to 1 mole trypsin) at low temperatures. The original method of Kunitz is also carried out at 0-5°, but with ratios of CHTG-A to trypsin of 2,000:1 and is called "slow" activation (41). The sequence of chemical changes which takes place during activation was described by two groups (8,42,43). A tryptic split between arginine-15 and isoleucine-16 is the first event, giving rise to CHT-A₁, a very unstable form which quickly undergoes chymotryptic digestion between leucine-13 and serine-14, liberating the dipeptide Ser-Arg, to form CHT-A₂.

Slow activation proceeds over a period of days with the same initial tryptic hydrolysis generating chymotryptic activity, but ~~two~~ ^{three} chymotryptic splits occur. A second dipeptide, Thr-Asp (NH₂), is produced after breakage of bonds between tyrosine-146 and threonine-147, and between asparagine-148 and alanine-149 to give the chymotrypsins A₃ and the final stable form CHT-A₄ (α chymotrypsin). CHT-A₄ is readily

crystallized and was one of the first enzymes obtained in a highly purified form.

It is likely that fast activation is the physiologically important mechanism because of the high concentration of trypsinogen found normally associated with the zymogens in pancreatic juice, so a study of the activation of CHTG-B would yield most significant information if concentrated upon the fast method. To date, three reports have appeared describing this process in CHTG-B. Kassell and Laskowski (44) reported that an initial tryptic digestion cleaved the Arg-Ile bond as in CHT-A₁, and Guy et al. (27) followed this up by isolating and characterizing the A' chain of CHT-B* after rupture of the disulfide bonds with performic acid. During this work, it was found that alanine replaced serine at residue 14 and that there was no form of CHT-B analogous to CHT-A₂ in that the Leu-Ala bond (residues 13-14) was not split and no activation dipeptide was produced. These authors suggest that the substitution of alanine for serine in this position may be responsible for blocking the cleavage.

A report has recently come from Laskowski's laboratory (Krehbiel et al. (16)) describing the activation of CHTG-B at 37°. It was found, as is substantiated in a later chapter, that the rate of activation is considerably higher than for CHTG-A. In all the experiments described, high ratios of trypsin to CHTG-B were used, generally about 1:10, and in

*The A' chain of CHT-B is equivalent to the A chain of CHT-A₄ plus the dipeptide Ala-Arg.

some instances CHTG-B:trypsin reached 1:3 without apparently showing any adverse effect upon the chymotryptic activity produced. For the determination of new terminal residues produced, the zymogen was activated in the presence of 10^{-5} M indolepropionic acid and 0.01 M CaCl_2 to minimize chymotryptic cleavages. However, the authors report, in addition to 0.5 mole of isoleucine, 0.25 mole alanine, 0.12 mole threonine, 0.11 mole lysine, and 0.16 mole serine as new N-terminal groups. A maximum of 0.7 mole of C-terminal arginine was found, which decreased with time of activation, while the enzyme was not examined for new non-basic C-terminal groups. Thus the earlier report of a split between arginine and isoleucine was confirmed and there were indications from the submolar release of new N-terminal residues that further chymotryptic splits occurred.

The activation described by the above two groups of authors gives rise to a form of CHT-B analogous to the CHT-A₁ of Bettelheim and Neurath (42). CHT-A₁ was shown to be unstable, undergoing autolysis to CHT-A₂. While Guy et al. were unable to find a form of CHT-B analogous to CHT-A₂, the loss of C-terminal arginine and the reduction in esterase activity with increasing activation time reported by Krehbeil et al. point to there being other forms of CHT-B produced by chymotryptic autolysis. As yet, no attempt has been made to demonstrate these changes or describe them in terms of the primary structure of the protein.

The evidence for CHTG-A undergoing a shape change on conversion to CHT-A is derived from ultraviolet spectrophoto-

metry, ultracentrifugation and optical rotatory dispersion. Chervenka (45) showed that the absorbance of CHT-A at 287 m μ was less than that of CHTG-A. This change was ascribed to a shift in the spectrum of tyrosine to shorter wavelengths brought about by an alteration in the tertiary structure of the molecule. Hashizume and Imahori (39) obtained similar spectral changes after activation of CHTG-A with pronase.

While the original observation of Schwert (86) that CHT-A₄ has a greater tendency to dimerise than has CHTG-A is well-substantiated (89,90), reported changes in optical rotatory dispersion during activation appear contradictory. Neurath et al. (49) made the initial observation that the activation of CHTG-A was accompanied by a decrease in laevo-rotation at 589 m μ . Later, more extensive experiments by Imahori et al. (48) showed the percent helix of CHTG-A to be 12% and that of CHT-A₁ to be 23%. However, recent figures quoted by Schellman and Schellman (50) from measurements made in both the ultraviolet and visible regions of the spectrum indicate no difference in the helical contents of CHTG-A and CHT-A.

Two groups of workers (12,24) have demonstrated that during activation of CHTG-A, the isoelectric point changes from 9.1 (for CHTG-A) to 8.3 (for CHT-A₂); Kubacki et al. (12) have also shown that the B system undergoes a similar change, from 5.2 (for CHTG-B) to 4.7 (for CHT-B). The increase in acidity from CHTG-A to CHT-A₂ may be readily ascribed to the loss of the basic Ser-Arg dipeptide, but none of the published data on CHTG-B activation demonstrates a similar basic peptide

release in molar quantities, although a search for such a peptide has been reported but not described in detail (16). Thus at this time, there is no simple explanation for the observed difference in the isoelectric points of CHTG-B and CHT-B.

A continuing program of inquiry into the structure and function of the CHT-B system has been instituted in this laboratory. The first phase of the investigation covered the isolation and properties of CHTG-B, with an analysis of the specificity of CHT-B. The findings were incorporated into a thesis by A. G. Enenkel (23). This second phase describes the physical and chemical changes taking place during the production of CHT-B from CHTG-B.

II. KINETICS AND INHIBITION OF CHYMOTRYPSIN-B

The purpose of the present work was to elucidate the chemical and physical changes accompanying activation of CHTG-B. Since some of these changes could be associated with the initial tryptic attack, while others could arise from subsequent autolytic proteolysis by the generated chymotrypsin, it was desirable to have available a method suitable for rapid and irreversible inhibition of chymotryptic activity. The classical reagent for this purpose is DFP as used by Bettelheim and Neurath (42), Dreyer and Neurath (43) and Röver et al. (88) in their studies on the activation of CHTG-A. However, early in this work it was observed that the efficiency of inactivation of CHT-B by DFP was less than for CHT-A. A search was therefore made for a more efficient reagent for the inhibition of the B enzyme. Towards this end, a number of reagents have been examined and their kinetic constants for the CHT-A and CHT-B systems compared. The reagents may be conveniently classified into: (a) irreversible inhibitors, (b) competitive inhibitors, and (c) acylating agents.

In any study of an enzyme system, it is necessary to have available a simple but accurate means of measuring enzyme activity. Chymotryptic activity has been measured in many ways, using natural substrates (various proteins), synthetic ester and amide substrates, or acylating agents. The two methods used routinely in this work fall into the last

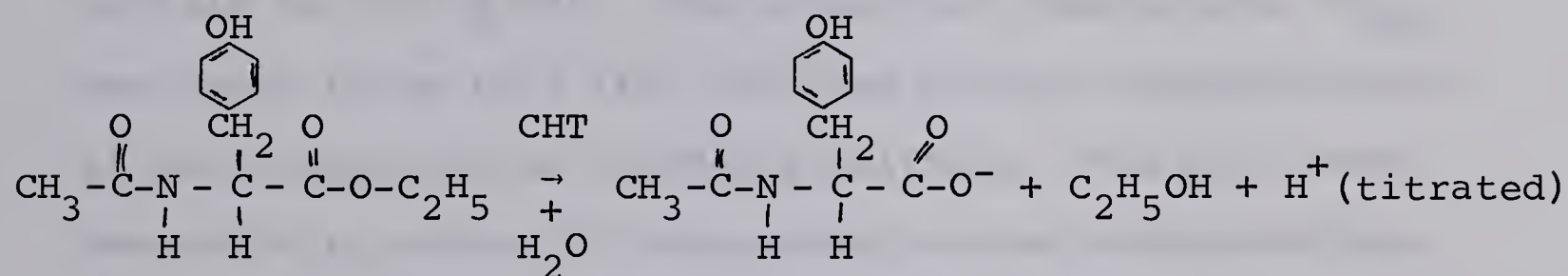
two categories and depend upon quite different principles.

The "rate assay" method is based upon measurements of the zero order rate constant for hydrolysis of a specific substrate by the enzyme. For both CHT-A and CHT-B, the ester substrate ATEE was chosen. Routine estimation of the zero order rate constant, expressed as k' (meg substrate/ml hydrolysed per min per mg enzyme N/ml) was carried out on solutions of 10 mM ATEE in 0.01 M Tris-HCl, pH 8.0, 0.05 M CaCl_2 , 0.1 M KCl. The salts were included in the assay system because they have been shown to increase and stabilize the esterase activity (see Chapter III). Rupture of the ester bond of the substrate exposes a carboxyl group which, at pH 8, is fully ionized so that one equivalent of H^+ ion is released for each equivalent of substrate hydrolysed. Maintenance of the pH at 8.0 by titration of the released H^+ with dilute base of known molarity results in a linear base uptake with time which is directly related to the amount of substrate hydrolysed.

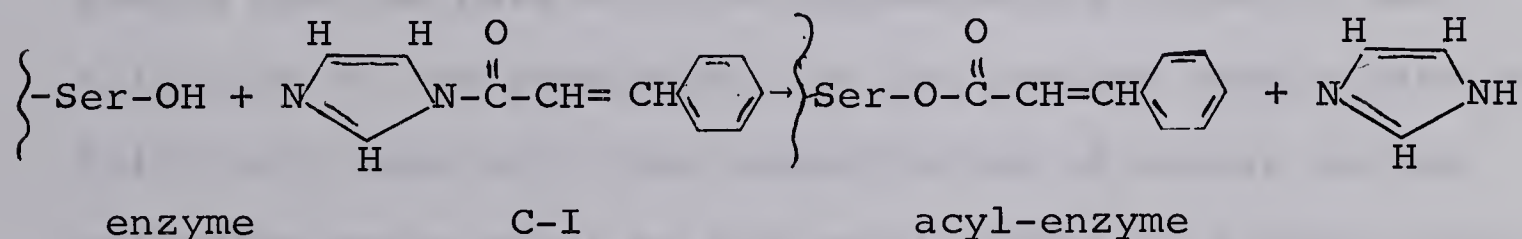
Schonbaum et al (58) first described the use of imidazolium compounds to acylate CHT-A₄, and devised a system for quantitative estimation of the enzyme. The imidazolium reagents are capable of reacting with the active serine residue in the enzyme to form an acyl enzyme. Cinnamoylimidazole (C-I) was first used for titration of CHT-A₄ at pH 5 as the rate of hydrolytic destruction of C-I at this pH was low and the deacylation rate of cinnamoyl-CHT-A₄ (C-CHT-A₄) was also relatively slow, while the acylation reaction was extremely fast. The absorption spectra of C-I and C-CHT-A₄ are well-separated so that, at a wavelength of 335 mμ, C-CHT-A₄ has essentially no absorption while C-I has an ϵ of 9.3×10^3 . Thus, a drop in A_{335} of a solution of C-I

upon addition of CHT-A₄ represents a loss of C-I as C-CHT-A₄ is produced. The magnitude of the drop is then related to the molar extinction: (ϵ) of C-I. The percent purity of the enzyme may be calculated by comparing the molar concentration given by C-I titration with the molar concentration of protein calculated from A₂₈₀. Provided that suitable assay conditions can be found (i.e. where the destruction rates of acyl imidazole and acyl enzyme are slow, acylation is fast, and an appropriate wavelength where changes in absorption can be related to the loss of acyl imidazole or the formation of acyl enzyme), any convenient acylating agent may be used.

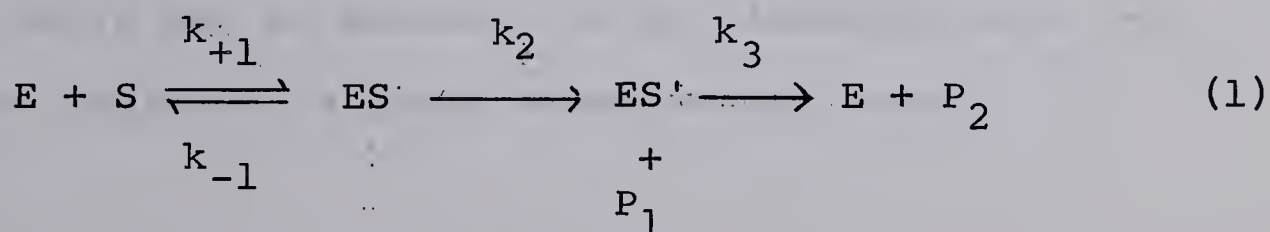
The reaction occurring with each method can be summarized : (a) rate assay:



(b) acylation:



It can be seen that the methods measure different parameters. The rate assay is essentially k_3 in equation (1) while the "all or none" acylation technique measures the total concentration of active sites.



Ideally, of course, they should be directly comparable, provided that k_3 is rate limiting and is the same for all forms of CHT-B. This is certainly not the case for CHT-A₁ and CHT-A₄ and, as later experiments will show, is also not true for different forms of CHT-B. Thus it is theoretically possible to obtain variable rate assay results from enzyme preparations giving identical values for the active site concentration measured by the "all or none" acylation technique.

Determination of zymogen or enzyme concentration throughout this work has been made generally from measurement of the absorption at 280 mμ of a dilute solution of protein in 10^{-3} M HCl. The extinction coefficient, $E_{1\text{cm}}^{1\%}$, was found to be 18.7 (15), so that protein concentrations in the range 0.01 to 0.02% are suitable. The main weakness of this method of determining enzyme concentrations is that two major conditions must be met. The protein sample must be pure with no contaminating material contributing to the absorbance and the zymogen sample must be fully activated with the concentration of active enzyme molecules being equal to the total number of protein molecules. Both these conditions appear to be satisfied for CHT-B. The preparative procedure has been shown to give, routinely, nearly homogenous CHTG-B which is 95% activatable (17). Thus measuring enzyme concentration by this means, while not as accurate as by titration with C-I, does not introduce a large experimental error.

Alternatively, the acylation procedure described above could be used for CHT-B where high concentrations of the enzyme are available. The major disadvantage of the acylation technique is the relatively large amount of protein required (e.g. 0.1 μ mole as opposed to 2×10^{-4} μ mole for rate assay). Also, the rate assay is sensitive to changes in catalytic efficiency, allowing detection of different forms of CHT-B. Thus for the purposes of this study, the rate assay, combined with measurements of A_{280} , was the most suitable method of estimating enzymic activity.

The CHT-B used in all the experiments described in this chapter was prepared by activating a 1% solution of CHTG-B at pH 8.0 and 0° with a CHTG-B : trypsin ratio of 40:1 for 1 hour. The activation solution was then brought to pH 3.0 with 6 N HCl, dialysed exhaustively against 10^{-3} M HCl and freeze-dried. The zymogen was prepared from beef pancreas glands by the method of Enenkel and Smillie (15).

1. Kinetic constants for ATEE hydrolysis

Before embarking upon a comparison of the competitive inhibitors of CHT-B, it is necessary to know the kinetic constants for hydrolysis of a suitable substrate. CHT-A₄ has been shown to hydrolyse a wide range of synthetic substrates (51,55), one of the most popular being ATEE. Since this substrate is used routinely in this and other laboratories, and

the kinetic constants for its hydrolysis by CHT-A₄ are accurately known, K_m and k₃* were calculated for the ATEE-CHT-B system.

The most common method of evaluating enzyme kinetics is to determine the reaction rate over a range of substrate concentrations, followed by graphical analysis, according to one of the rearrangements of the Michaelis-Menten equation (54). As the limited solubility of ATEE makes the range of substrate concentration rather small for this treatment, an integrated form of the equation was used (52,54). Ideally, this method allows the calculation of both K_m and k₃ from a single experiment at one substrate concentration.

$$k_3 \text{ et} = K_m \ln \frac{S_0}{S_0-P} + P \quad (2)$$

which may be rearranged

$$\frac{2.3}{t} \log \frac{S_0}{S_0-P} = \frac{k_3 e}{K_m} - \frac{1}{K_m} \cdot \frac{P}{t} \quad (3)$$

where S₀ = initial substrate concentration; P = product concentration at time t; K_m = Michaelis constant, taken as [S] at half maximum velocity; e = enzyme concentration; and k₃ is defined by equation (1).

For equation (2) to be valid, the decrease in rate of

*Although the terms K_m and k₃ are used throughout this work, these are apparent values. According to Alberty (88), the pH effect on an enzyme reaction is a complex one involving ionization of enzyme and intermediate enzyme-substrate complexes. As not all ionic forms will react in the same way or at the same rate, the true values for K_m and k₃ may be very different from the apparent ones (see Sturtevant, 89).

enzyme action during hydrolysis must be due solely to a decrease in saturation of the enzyme as the substrate concentration falls (54). All measurements must therefore be made on the non-linear part of a progress curve where the reaction is no longer zero order. Equation (3) can be solved by plotting $2.3/t \log S_0/S_0-P$ versus P/t , producing a straight line of slope $-1/K_m$ with the intercept on the P/t axis equal to k_3e .

a. Method

Solutions of ATEE over a range of $1.6 \times 10^{-3} \text{ M}$ to $9.5 \times 10^{-3} \text{ M}$ were prepared in 0.01 M Tris-HCl, pH 8.0, 0.1 M KCl, 0.05 M CaCl_2 . For each assay, a 3.0 ml portion of substrate solution was pipetted into a thermostatted reaction vessel designed for small volumes (63). The vessel was used in conjunction with a Jacobsen-Leonis autotitrator (64) (Ole Dich, Copenhagen) and a Radiometer type TTT-1 titrator. The pH was adjusted to 8.0 with 0.2 N NaOH, and temperature maintained at 25° . To begin hydrolysis, $25 \mu\text{l}$ of a dilute ($\sim 5 \times 10^{-6} \text{ M}$) enzyme solution was introduced into the reaction mixture. The uptake of 0.2 N NaOH was recorded against time. The reaction was allowed to go to completion and the total equivalent of base required to maintain the pH at 8.0 was used to calculate the actual substrate concentration.

Two separate series of experiments were carried out using substrate concentrations of 1.62, 3.70, 5.82, 6.49 and $9.52 \times 10^{-3} \text{ M}$ in the first instance; and 3.76, 4.78 and $9.58 \times 10^{-3} \text{ M}$ in the second. A single enzyme solution was

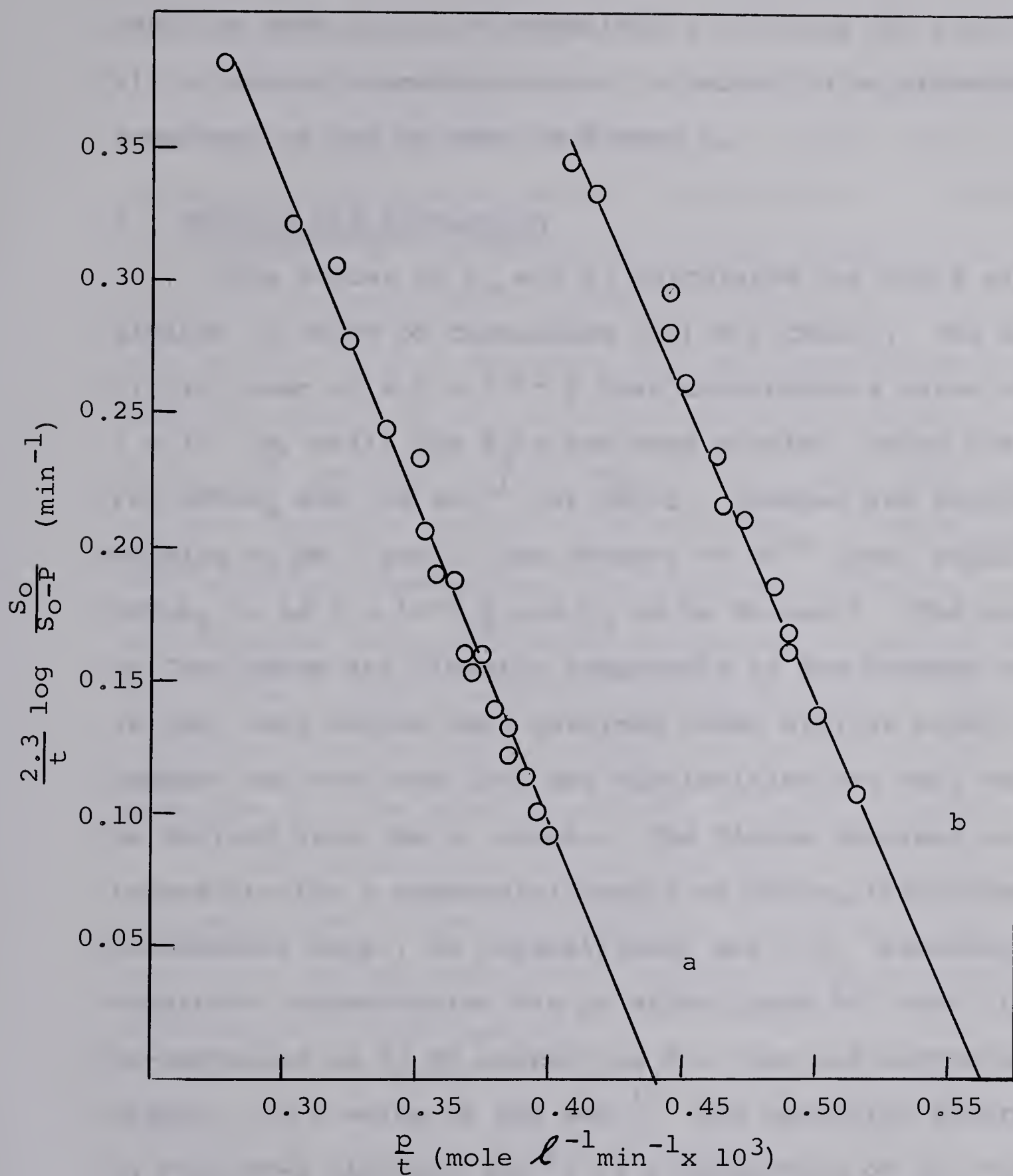


Figure 1. Graph of data from integrated Michaelis-Menten equation.

a) $e = 4.00 \times 10^{-8} \underline{M}$; slope $2.41 \times 10^3 \ell \text{ mole}^{-1}$.
 $K_m = 4.15 \times 10^{-4} \underline{M}$ $k_3 = 183 \text{ sec}^{-1}$.

b) $e = 4.35 \times 10^{-8} \underline{M}$; slope $= 2.38 \times 10^3 \ell \text{ mole}^{-1}$.
 $K_m = 4.2 \times 10^{-4} \underline{M}$ $k_3 = 215 \text{ sec}^{-1}$.

used for each series of experiments allowing the results from all substrate concentrations of a series to be presented together, as can be seen in Figure 1.

b. Results and Discussion

The values of K_m and k_3 calculated for CHT-B are quite similar to those of Cunningham (52) for CHT-A₄. The K_m is a little lower at 4.2×10^{-3} M than Cunningham's value of 7×10^{-3} M, while the k_3 's are very similar, being 204 sec^{-1} for CHT-A₄ and 199 sec^{-1} for CHT-B. Spencer and Sturtevant (53) working at pH 7 and in the absence of Ca^{++} ions, found K_m for CHT-A₄ to be 5×10^{-3} M and k_3 to be 96 sec^{-1} . The results of Cunningham are directly comparable to the present results in that both values were obtained under similar conditions. Support for the view that the similarities are real can also be derived from the k' values. The figure obtained in this laboratory for a commercial sample of CHT-A₄ (Worthington Biochemical Corp., 3x crystallized) was 3.3. Assuming that substrate concentration has no effect upon k' , then it may be re-expressed as k_3 by correcting for time and enzyme concentration, to a value of 220 sec^{-1} . The variation observed in k_3 for CHT-B ($183\text{--}215 \text{ sec}^{-1}$) is a reflection of the varying enzyme purity from one preparation to another. K_m , on the other hand, is independent of enzyme concentration or purity, as it may be defined as the substrate concentration at half maximum velocity in which no enzyme concentration term is found. It is therefore a much more reproducible value.

2. Competitive Inhibition

Previous workers on the activation of CHTG-A (42,43) have used 0.1 M β -phenylpropionate as a chymotryptic inhibitor to prevent or retard the destruction of CHT-A₁. This compound is an inconvenient reagent because high concentrations are necessary to produce a significant effect, and also because it is a strong inhibitor of carboxypeptidase-A (CP-A), having a K_i of 6.2×10^{-5} M for this enzyme (55). This would necessitate its removal on an anion exchange resin before C-terminal analysis could be made. Wallace et al. (67) have surveyed many competitive inhibitors of CHT-A₄ and their data indicate that both indole and benzo (f) quinoline are much better inhibitors than β -phenylpropionate. These three inhibitors were tested with CHT-B to find which would be most effective for the specific control of CHT-B activity.

The values of K_m and k_3 described in the previous section were used in the calculation of the K_i 's. These values, together with experimentally determined initial rates, can be substituted in the equation:

$$K_i = \frac{v_o \cdot K_m I}{k_3 e \cdot S_o - v_o (K_m + S_o)} \quad (4)$$

where v_o = initial rate; K_m , k_3 , e and S_o are used as in equation (3); and I is inhibitor concentration.

a. Method

Chymotryptic assay was carried out at 25° as described in section 1 of this chapter, using solutions of 10 mM ATEE

Table II
Competitive Inhibitors of Chymotrypsin-B

Inhibitor	[I] mole l^{-1}	K_i mole l^{-1}
β -phenyl propionate	8.52×10^{-2}	1.5×10^{-2}
	4.26×10^{-2}	2.0×10^{-2}
indole	4.08×10^{-3}	4.2×10^{-4}
	2.45×10^{-3}	4.9×10^{-4}
benzo (f) quinoline	3.50×10^{-5}	1.0×10^{-4}
	7.85×10^{-5}	1.1×10^{-4}

in 0.01 M Tris-HCl, pH 8.0, 0.1 M KCl and 0.05 M CaCl₂ in which were dissolved suitable concentrations of inhibitor. Inhibitor concentrations in the salt buffer solution were determined spectrophotometrically on samples diluted in ethanol prior to the addition of ATEE, using the data given in the following table (taken from reference 65):

Inhibitor	ϵ at $\lambda_{\max}$	$\lambda_{\max}$
Indole	6.31×10^3	280
β -phenylpropionate	2.51×10^2	258
benzo (f) quinoline	3.93×10^3	344

Indole and β -phenylpropionic acid were purchased from Eastman Kodak Co., and benzo (f) quinoline from British Drug Houses.

b. Results and Discussion

The agents tested must be evaluated as to their usefulness in controlling autolysis of CHT-B during the activation process. As both indole and benzo (f) quinoline have limited solubilities, this usefulness will depend not only on the K_i listed in Table II but also upon the solubility. On this basis, benzo (f) quinoline cannot be used for controlling autolysis as its maximum solubility is approximately 1×10^{-4} M, while a 4×10^{-4} M solution of zymogen is normally used in activation mixtures. Indole, on the other hand, is soluble enough to allow the preparation of a 2×10^{-2} M solution.

A comparison of the K_i values for indole and β -phenylpropionate shows that the K_i of the former is 40 times smaller than that of the latter. As indole solutions can be made of much higher (20x) concentration than enzyme, the inhibitor appears suitable for use in activation experiments. Finally, considering the additional factor that indole is without effect upon CP-A (see Appendix A) and therefore can remain in solutions used for C-terminal analysis, it is clear that indole is the preferred reagent for further use.

3. Irreversible Inhibitors

Any study of the structure of proteolytic enzymes is complicated by the risk of autolysis. It is therefore important to be able to prepare a stable inactive derivative of the enzyme. A suitable inactivator should possess (a) a high rate of reaction with the enzyme, with no reactivation under conditions used in subsequent experiments; (b) high solubility in aqueous media, allowing the use of large excesses; (c) specificity for the particular enzyme; and (d) non-toxicity and safety in handling. Two non-reversible inhibitors were examined for their effect on CHT-B. One, DFP, is well-known and its reaction rate with CHT-A₄ was calculated by Ooms (56); while the other, diphenylcarbonyl chloride (DPC), was recently described (57). In view of their rapid reaction with CHT-A₄, it was hoped that one of them would be useful with CHT-B.

Reaction rates of inhibitor with enzyme were measured in the following experiments, and the observed disappearance of enzyme activity substituted in the expression for a second

order reaction.

$$k = \frac{2.303}{t(I_0 - e_0)} \log \frac{e_0(I_0 - eI)}{I_0(e_0 - eI)} \quad (5)$$

where I_0 is inhibitor concentration, e_0 is enzyme concentration at 0 time, eI is inhibited enzyme at time t . The second order rate constant, k , may then be obtained by plotting $\log \frac{I_0 - eI}{e_0 - eI}$ versus t , to give a straight line of slope $\frac{k(I_0 - e_0)}{2.303}$.

a. Method

Reaction conditions for inhibition were chosen so that the residual enzyme could be assayed either in the pH-stat with ATEE or by titration with the acylating agent anisoyl imidazole (A-I). A 1.09 M stock solution of DFP was prepared by quantitative transfer with isopropanol of 1 g of DFP from a sealed vial (supplied by Mann Research Laboratories) to a 5 ml volumetric flask and adjusting to volume with isopropanol. Suitable dilutions in isopropanol were then prepared. Samples of DPC (Eastman Kodak Co.) were weighed and dissolved in ethanol.

(i) Assay with pH-stat

A 1.0 ml volume of enzyme (5×10^{-6} M) was prepared in 0.1 M Tris-HCl, pH 8.0 and 25 μ l removed for assay against ATEE as described in part one of this chapter. To the remaining solution, incubated at 25^o, was added 50 μ l of 5×10^{-4} M DFP solution or 50 μ l of 1.94×10^{-4} M DPC solution. Samples of the mixture were withdrawn at time intervals for assay against ATEE.

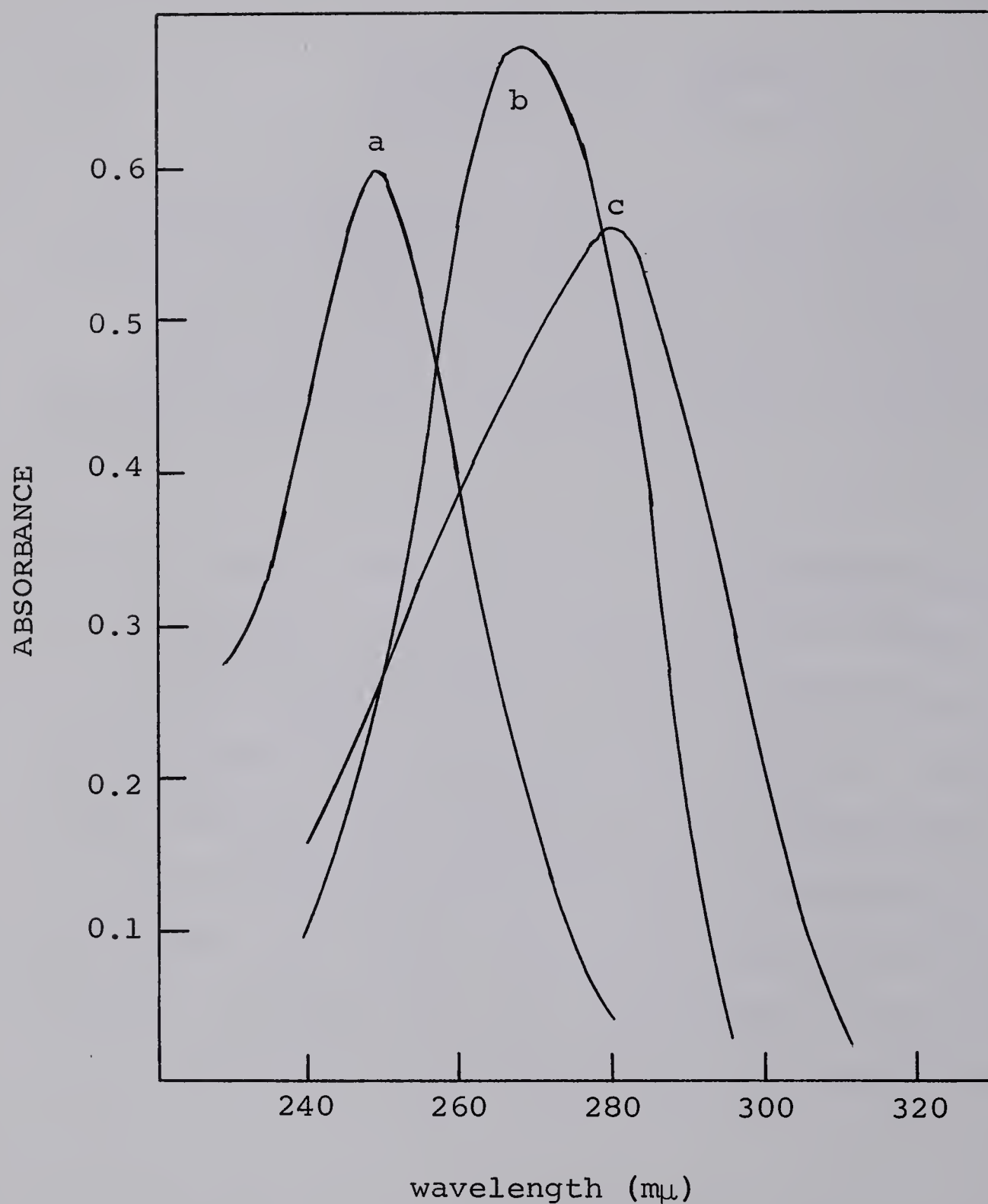


Figure 2. Absorption spectra of p-anisic acid, A-I and difference spectrum of anisoyl-CHT-B versus CHT-B. All measurements made at 25° in 0.1 M phosphate buffer pH 8.

- a) p-anisic acid $\epsilon_{\text{at } \lambda_{\text{max}}} = 11.9 \times 10^3$
- b) anisoyl-CHT-B $\epsilon_{\text{at } \lambda_{\text{max}}} = 13.6 \times 10^3$
- c) anisoylimidazole $\epsilon_{\text{at } \lambda_{\text{max}}} = 11.4 \times 10^3$

(ii) Assay by Titration

The principle of spectrophotometric titration of an enzyme with an acylating agent has been outlined at the beginning of this chapter. A-I was chosen for use in this experiment because the stability of both A-I and anisoyl-CHT-B (A-CHT-B) is high at pH 8. Further, as can be seen from the spectra in Figure 2, a wavelength of 310 m μ is outside the range of absorption of A-CHT-B while the extinction of A-I remains high. Therefore, the magnitude of a drop in A_{310} upon addition of enzyme to A-I can be used to calculate the concentration of A-CHT-B. The experiments were performed with a Beckman DK-2 ratio recording spectrophotometer with 1 cm light path silica cells in both sample and reference beams; the cell housing was thermostatted to 25 $^{\circ}$ and the wavelength set at 310 m μ . A preliminary blank determination of the absorbance of A-I was performed by adding 25 μ l A-I (2×10^{-2} M, in acetonitrile) to the sample cell when both the cells contained 3.0 ml 0.1 M Tris-HCl buffer, pH 8, and 25 μ l ethanol; A_{310} was recorded. The ethanol was included in the solution to act as a blank for the DPC solution added in later experiments. All microlitre volumes of reagent were added to the cells on the end of a small stirring rod.

Zero time assay of CHT-B was next performed by adding 25 μ l of A-I to the sample cell containing 3.0 ml of 5×10^{-5} M enzyme in 0.1 M Tris-HCl, pH 8.0 and 25 μ l ethanol, while the reference cell contained buffer and ethanol as before. Once more the A_{310} was recorded.

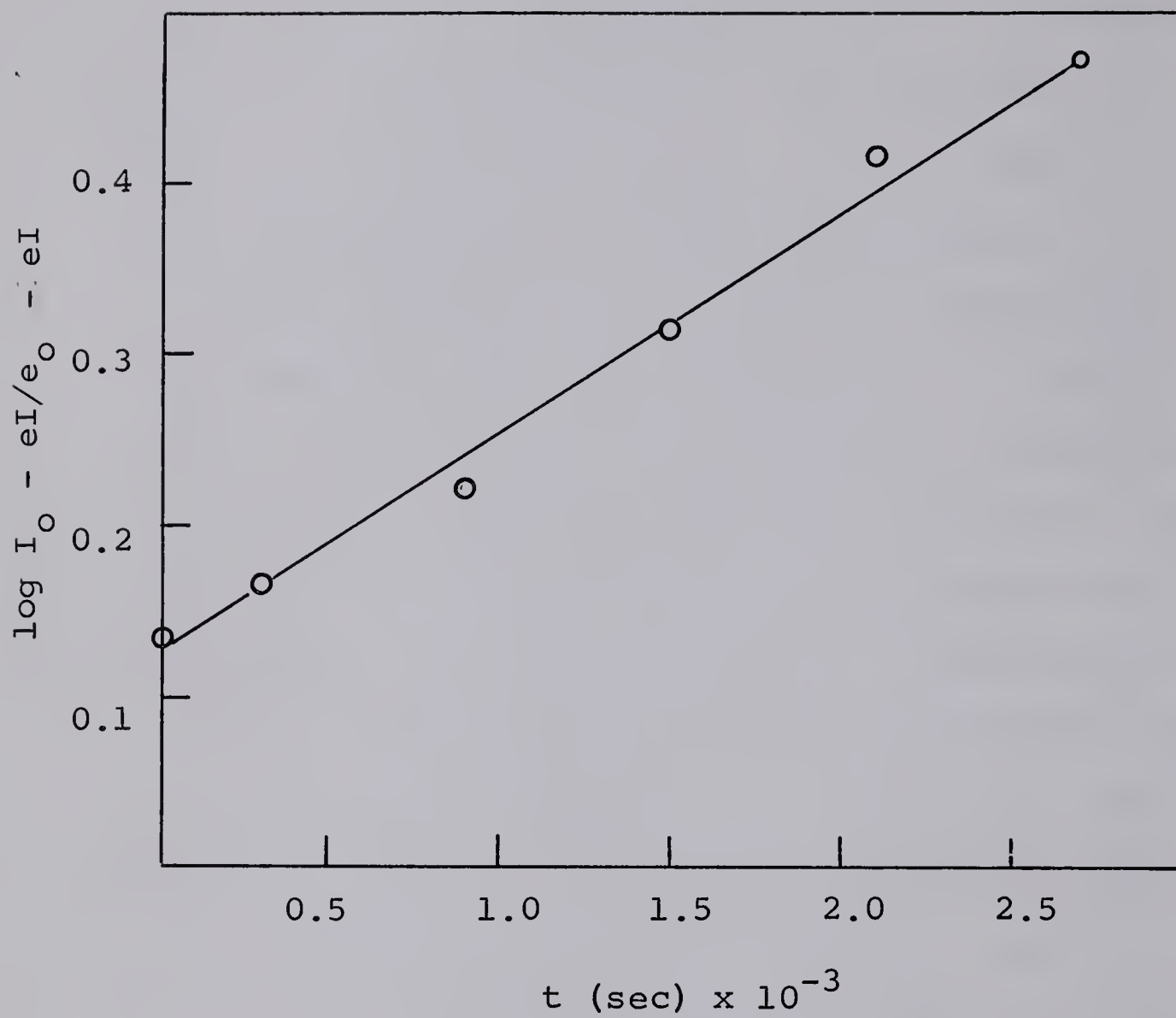


Figure 3c. Irreversible inhibition of
 CHT-A₄ by DPC.

$$k = 556 \text{ } \ell \text{mole}^{-1} \text{ sec}^{-1}$$

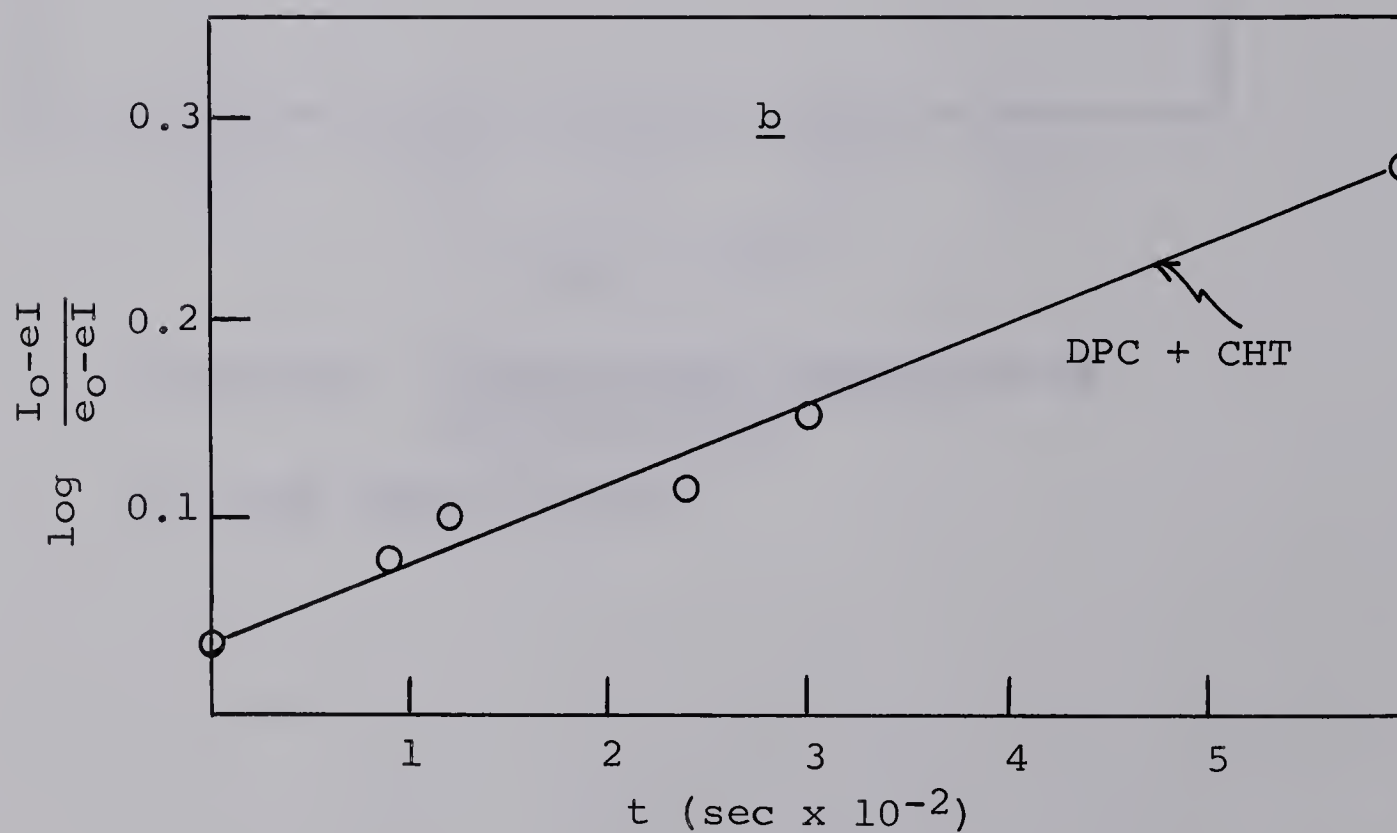
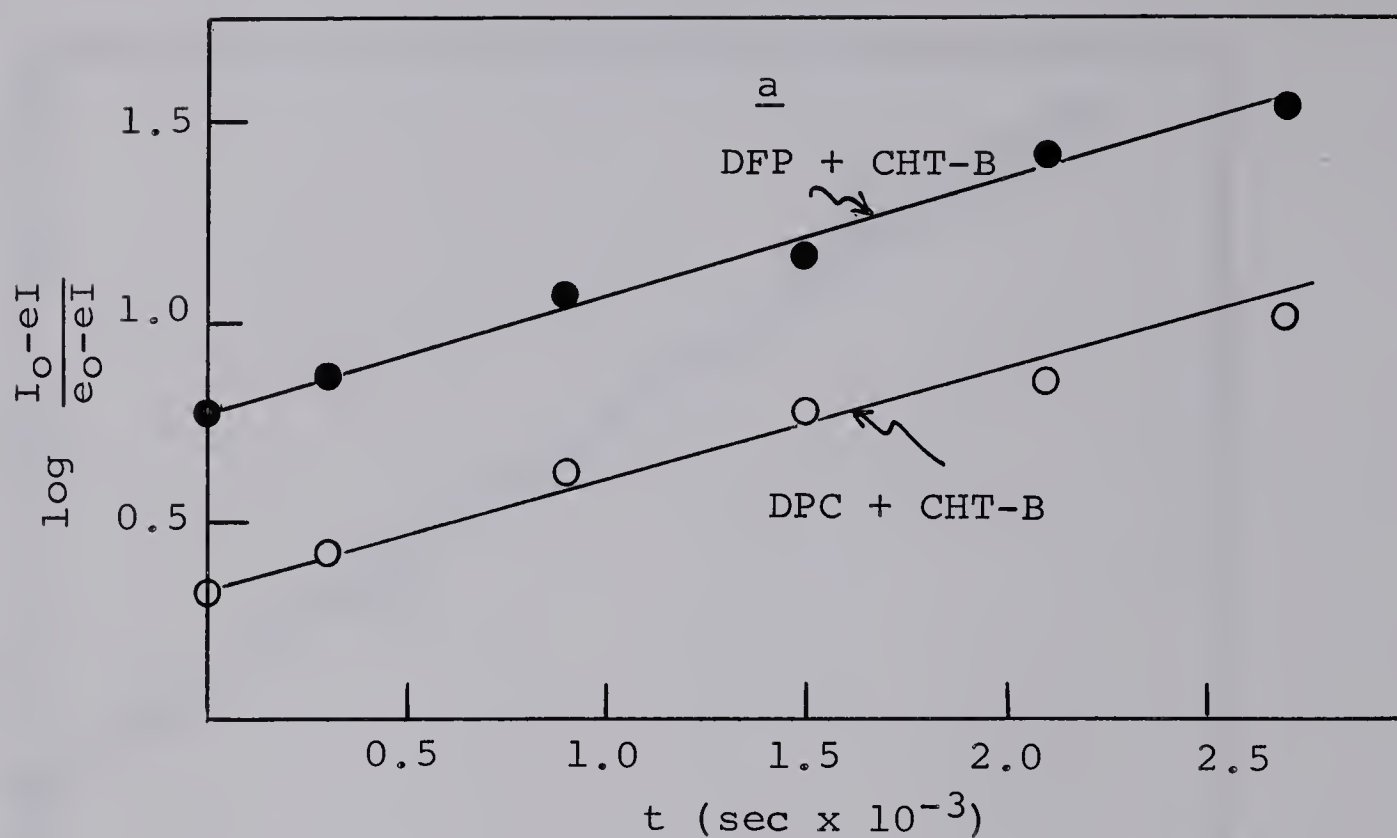


Figure 3. Irreversible inhibition of CHT-B.

a) Using rate assay.

b) Using titration assay.

For the time study on DPC effect, the sample cell contained 3.0 ml enzyme solution plus 25 μ l of 1.0×10^{-2} M DPC in ethanol. The inhibition reaction was allowed to continue for the desired period before residual enzyme was titrated with A-I. Further samples of enzyme were reacted with DPC for varying periods before titration with A-I until a sufficient number of determinations had been made to allow graphical analysis.

b. Results and Discussion

The results of the rate determinations bear out early qualitative observations made in this laboratory that DFP reacts more slowly with CHT-B than with CHT-A₄. Ooms (56) reported the second order rate constant for the reaction of DFP with CHT-A₄ as $317 \text{ l mole}^{-1}\text{sec}^{-1}$, while the value with CHT-B is $35 \text{ l mole}^{-1}\text{sec}^{-1}$, almost ten times slower. Similarly the rate constant for the DPC-CHT-A₄ reaction is $610 \text{ l mole}^{-1}\text{sec}^{-1}$ and with DPC-CHT-B, the observed rate is $122 \text{ l mole}^{-1}\text{sec}^{-1}$. The figures for DFP inhibition of CHT-B and A are closely comparable, as both experiments were at a similar pH (7.7 for CHT-A₄, 8.0 for CHT-B), but there may be a wider variation in the results for DPC, as those reported for CHT-A₄ were at pH 7.0 in the presence of 0.1 M CaCl₂. Nevertheless, the fivefold decrease in rate is indicative of CHT-B's resistance to inhibition.

A calculation, using these constants, can be made of the time required to inactivate a sample of CHT-B at 25°. The solubility limit of DPC in 10% ethanol is 1×10^{-4} M;

using this concentration and 1×10^{-4} M enzyme, a residual activity of 1% would take two hours to achieve. Using a 1×10^{-3} M solution of DFP, which is more soluble than DPC, and 4×10^{-4} M enzyme (1%), 1% of the enzyme activity would remain after 3 minutes. While the reaction rate is satisfactory at 25° , at 5° a period of 10-15 minutes would be required and during this time a considerable amount of autolysis may take place. DPC is clearly not suitable for routine inactivation of 1% protein solutions, and DFP reacts too slowly to be used in terminal analysis, although the rate of inhibition is high enough for the preparation of inactive enzyme when a small amount of autolysis is of no importance.

4. Acylating Agents

The unsuitability of DFP for use in C-terminal analysis of CHT-B resulted in a widening of the search for suitable inhibitors and attention was turned to the imidazolium derivatives described by Schonbaum et al. (58). A number of reports have appeared describing the properties of various acyl derivatives of CHT-A₄ and other "serine" esterases (60, 61). These results, together with information from the breakdown of other synthetic substrates, have recently been used to support a detailed description of a proposed mechanism of CHT-A₄ catalysis (62). Although nothing of this scope has been attempted with CHT-B, C-I has been used for titration of the enzyme (15). It was therefore felt that another imidazolium derivative might be produced which would react quickly with CHT-B to inactivate the enzyme, and the acyl group, once

attached, would be removed slowly, thus providing an inactive acyl-CHT-B of some stability.

A literature survey indicated that two acyl imidazoles might prove useful. Bender et al. (66) used p-nitrophenyltrimethylacetate (NTMA) to study the deacylation rate of trimethylacetyl-CHT-A₄, and found the deacylation rate to be quite slow at pH 8, with a first order rate constant of $1.5 \times 10^{-4} \text{ sec}^{-1}$. The low rate is probably the result of steric hindrance by the methyl groups, and should allow the production of trimethylacetylimidazole (TMA-I) which would be fairly resistant to hydrolysis at pH 8. Caplow and Jencks (60) carried out a survey of various substituted benzoyl-CHT-A₄ derivatives, and their data indicated that p-methoxybenzoyl-CHT-A₄ had a similarly low deacylation rate ($k = 6.7 \times 10^{-4}$ at pH 8). Therefore, p-methoxybenzoylimidazole (anisoylimidazole, A-I) was also investigated. As no data were available on the stability of either imidazolium derivative at pH 8 and 25^o, the hydrolysis rates were measured. The reaction of C-I with CHT-B was also followed to compare the results with those already obtained for CHT-A₄. All three acyylimidazoles react very quickly with CHT-B, so this step was not examined.

a. Materials and Methods

(i) Preparation of Acylating Agents

C-I was purchased from Nutritional Biochemicals Corporation. A-I and TMA-I were prepared according to the method of Schonbaum et al. (58). A solution of ~10 mmoles acylchloride (anisoylchloride supplied by Eastman Kodak, and

trimethylacetyl chloride by K and K Laboratories), dissolved in 20 ml of dry benzene, was added dropwise from a separatory funnel into a stirred suspension of ~ 20 mmoles imidazole (Eastman Kodak) in 80 ml dry benzene, over a period of 10-15 minutes. The temperature of the suspension was maintained at 0° with an ice-water bath. Dry benzene was prepared by distillation of reagent grade (Fisher ACS) benzene, after discarding the initial azeotropic mixture. About 5 minutes after the addition of the acylchloride was complete, the temperature of the mixture was raised to approximately 25° , 30 ml of dry benzene added, and the stirring continued for a further 20 minutes to ensure redissolution of any precipitated acylimidazole. Imidazolium chloride was removed by filtration on a sintered glass funnel and the solution transferred to a flash evaporator for removal of benzene.

TMA-I was taken up in a minimum volume of acetonitrile (Eastman Kodak, spectro grade) and used without recrystallization. A-I was recrystallized from hot cyclohexane with a final yield of 65% (m.p. 69° , literature value $69-70^{\circ}$, (60)).

NTMA was prepared according to the method of Bender et al. (66). Five g (30 mmole) of p-nitrophenol (Fisher Scientific Co., reagent grade) was dissolved in 20 ml pyridine (Fisher, reagent grade) and added dropwise to 3 g (25 mmole) of trimethylacetylchloride at room temperature, with vigorous stirring. The solution was then warmed on an electric hot-plate before being quickly poured into a stirred ice-water mixture. NTMA was filtered off and washed, on the filter, with water and 0.1% sodium bicarbonate, before crystallization

from hot 95% ethanol (m.p. $95-97^{\circ}$, literature value $94-95^{\circ}$, (66)).

Stock solutions of A-I, C-I, and TMA-I in acetonitrile were kept at 5° in vessels with standard taper stoppers sealed with parafilm. Crystalline C-I and A-I, in screw-top bottles, were stored within larger bottles containing anhydrous calcium chloride, while NTMA was kept in a desiccator.

(ii) Measurement of Acylimidazole Hydrolysis and Acyl-CHT-B Deacylation Rates

Successful rate measurements by spectrophotometry depend, among other things, on the choice of a suitable wavelength for following the reaction. Ideally, a wavelength should be found where the ΔA is caused by the disappearance of starting compound or by the appearance of product only, i.e., where the spectra do not overlap. The wavelength of $310\text{ m}\mu$, chosen to follow A-I hydrolysis, fulfils these conditions, as can be seen in Figure 2. Similarly, $335\text{ m}\mu$ and $245\text{ m}\mu$ were used to follow the hydrolysis of C-I and TMA-I respectively.

Hydrolysis rates were measured with 3.0 ml of the appropriate 0.1 M buffer in 1 cm light path silica cells placed in both sample and reference beams of the Beckman DK-2 spectrophotometer. The temperature of the cell housing was maintained at 25° by circulating water from a constant temperature bath. At zero time, $10\text{ }\mu\text{l}$ of $3 \times 10^{-2}\text{ M}$ acylimidazole in acetonitrile was added to the sample cell, and the decrease in absorbance recorded with time. First order rate constants were calculated from the slope of a graph of $\log A$ vs t .

The deacylation rates of A-CHT-B and C-CHT-B may be measured directly after preparing the acyl-enzyme by addition

of acylimidazole to enzyme because the acyl-enzyme has a discrete absorption spectrum well-separated from that of the acid product. Reference to Figure 2 illustrates that provided the solution contains a small excess of enzyme, all of the A-I is used up in the formation of A-CHT-B so that any absorbance at 286 mμ is due to acyl-enzyme alone when measured against a reference cell containing an identical concentration of enzyme. This wavelength is outside the absorption band of p-anisic acid, allowing the decrease in A_{286} to be used as a direct measure of deacylation of A-CHT-B. Deacylation of C-CHT-B can be followed at 310 mμ for similar reasons. Both the reference cuvette and the sample cuvette contained 3.0 ml of 3×10^{-5} M enzyme. To begin the reaction, 50 μl of 1.7×10^{-3} M acylimidazole was added to the solution (final concentration, 2.7×10^{-5} M).

A different technique must be used to follow the deacylation of TMA-CHT-B, as this acyl-enzyme absorbs in the region below 240 mμ. Reaction with NTMA according to the method of Bender et al. (66) provides a suitable alternative. In this method, the increase in colour at 400 mμ resulting from the release of p-nitrophenol on addition of NTMA to CHT-B is measured. The release occurs in two stages, an initial fast reaction during which the enzyme is acylated, followed by a much slower release resulting from the newly deacylated CHT-B reacting with fresh NTMA. As the NTMA is present in excess over CHT-B and the rate of acylation is much faster than the release of enzyme by deacylation, the second part of the curve is a measure of the deacylation rate of TMA-CHT-B and

Table III
Decomposition Rates of Acylimidazoles

pH	A-I x 10 ⁴ sec ⁻¹	TMA-I x 10 ³ sec ⁻¹	C-I x 10 ⁴ sec ⁻¹
3.08 ^a	67	-	-
4.11 ^b	35	-	-
5.12 ^b	10	-	-
6.13 ^c	3.2	-	-
7.12 ^c	2.2	4.3	1.3
8.05 ^c	2.4	3.1	3.3
8.0 ^d	13	3.0	-

^a 0.1 M glycine-HCl

^b 0.1 M acetate

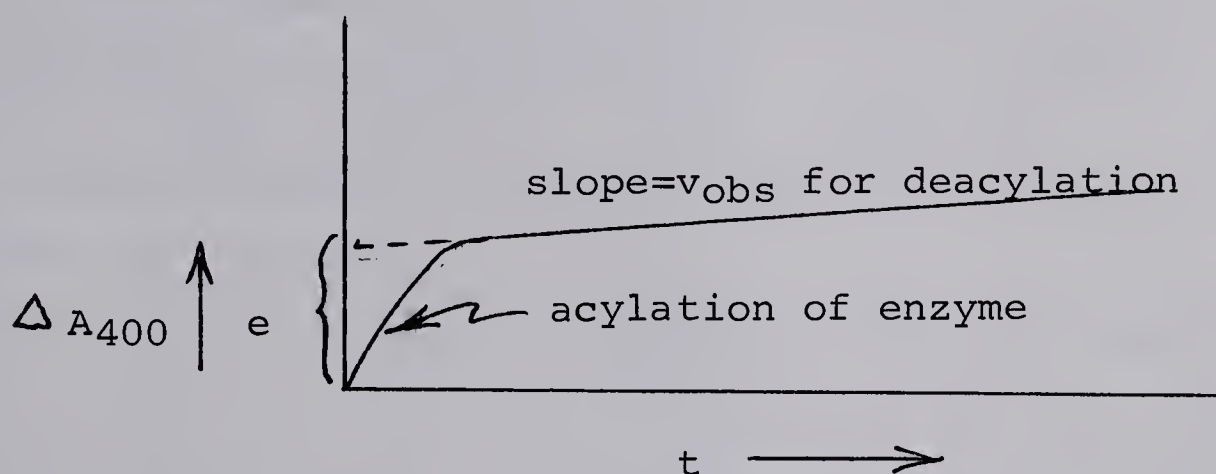
^c 0.1 M phosphate

^d 0.1 M Tris-HCl

can be analysed by the expression:

$$v_{\text{obs}} = k e \quad (6)$$

where v_{obs} is the rate of p-nitrophenol production, k is the first order rate constant for deacylation, and e is the acyl-enzyme concentration. Acyl-enzyme concentration is found by measuring the zero time intercept of the extrapolated straight line portion of the p-nitrophenol release curve. The $\epsilon_{400\text{m}\mu}$ for p-nitrophenol at pH 8.3 is 1.32×10^4 (65), and at pH 7.2 was calculated to be 9.45×10^3 .



Rates were measured at 25° with a wavelength of $400\text{ m}\mu$ and 3.0 ml of appropriate buffer in both sample and reference cells. To each cell was added $100\text{ }\mu\text{l}$ of $2 \times 10^{-3}\text{ M}$ NTMA dissolved in acetonitrile. At the start of the reaction, $50\text{ }\mu\text{l}$ of $2 \times 10^{-3}\text{ M}$ CHT-B was placed in the sample cell and the increase in A_{400} recorded.

b. Results and Discussion

As the C-terminal analyses with CP-A were carried out at pH 7-8, a suitable acylating agent for use in these experiments must be fairly stable in this pH range. Table III shows

Table IV

Deacylation Rates of Acyl-CHT-B's

Anisoyl-		Trimethylacetyl-		Cinnamoyl-	
pH	$k \times 10^4$ *	pH	$k \times 10^4$	pH	$k \times 10^2$
6.03 ^b	0.88	7.17 ^b	5.1	5.11 ^a	0.14
7.05 ^b	1.8	8.26 ^b	7.3	7.15 ^b	4.8
8.0 ^b	2.5	-	-	8.11 ^b	6.8
8.0 ^c	4.8	-	-	-	-

* k = first order rate constant in sec^{-1} .

^a 0.1 M acetate

^b 0.1 M phosphate

^c 0.1 M Tris-HCl

the first order rate constants of the three acylimidazoles tested. Evidently A-I and C-I are much more stable than TMA-I, A-I having a half-life of 48 minutes at pH 8, while the half-life of TMA-I is 3.7 minutes at the same pH. Thus, on the basis of acylating agent stability alone, the choice rests between A-I and C-I.

The second factor governing the choice of reagent is the stability of the acyl-enzyme. The first order rate constants for deacylation of the various acyl-enzymes are listed in Table IV. Again, the first two of the acyl derivatives are more stable than the third, but this time, it is the cinnamoyl derivative (C-CHT-B) which is labile with a half-life of approximately 10 seconds, compared to a half-life of 46 minutes for A-CHT-B₂.

The logical choice of acylating agent must therefore be anisoylimidazole, as both the agent and the acyl-enzyme are quite stable at pH 8. Digestion with CP-A was generally carried out for 60 minutes at pH 8 and 25⁰. In this time, more than half of the acyl-enzyme could be deacylated, so an excess of A-I must be present at the end of 60 minutes. To maintain this concentration of A-I, the initial addition must be in large excess over the enzyme. In practice, a fivefold excess was used; thus, even at the end of 60 minutes, there is a twofold excess of A-I over A-CHT-B.

5. General Discussion

Although the primary object of this chapter was to find suitable inhibiting agents for use with CHT-B in later

Table V

Some Kinetic Parameters of CHT-B₂ and CHT-A₄

Parameter	pH	CHT-B	CHT-A ₄	Units
K _m (app) for ATEE hydrol. ^a		4.2	7.0	mole l ⁻¹ x 10 ⁴ sec ⁻¹
k ₃ (app) " " "		199	204	
K _i for indole		4.5 ^a	8.0	mole l ⁻¹ x 10 ⁴ mole l ⁻¹ x 10 ² mole l ⁻¹ x 10 ⁴
K _i for β-phenylpropionate		1.8 ^a	2.5	
K _i for benzo(f)quinoline		1.0 ^a	0.63	
k(2nd order) for DFP		34, 37 ^a	317 ^c (56)	l mole ⁻¹ sec ⁻¹
k(2nd order) for DPC		124, 120 ^a	610 ^d (57)	l mole ⁻¹ sec ⁻¹
k(1st order) for deacylation of anisoyl-enzyme	6.03	8.8 ^f	0.50 ^f	sec ⁻¹ x 10 ⁵
	7.05	1.8 ^f	0.40 ^f	sec ⁻¹ x 10 ⁴
	8.0	2.5 ^f	0.67 ^f	sec ⁻¹ x 10 ⁴
	8.0 ^e	4.8 ^e	~ 2.5 ^e	sec ⁻¹ x 10 ⁴
k(1st order) deacylation of TMA-enzyme	7.17	5.1 ^f	1.1	sec ⁻¹ x 10 ⁴
	8.26	7.3 ^f	1.5	sec ⁻¹ x 10 ⁴
k(1st order) deacylation of cinnamoyl-enzyme	5.11	1.4 ^g	-	sec ⁻¹ x 10 ³
	7.15	4.8 ^f	0.65	sec ⁻¹ x 10 ²
	8.11	6.8 ^f	1.15	sec ⁻¹ x 10 ²

* Figures in parentheses are references.

^a pH 8.0, 25^o, 0.05 M CaCl₂^b pH 7.9, 0.1 M KCl^c 0.1 M phosphate, pH 7.7^d 0.01 M Tris-maleate, CaCl₂, pH 7.0^e 0.1 M Tris-HCl^f 0.1 M phosphate^g 0.1 M acetate^h 0.1 M borateⁱ 0.061 M phosphate

experiments, it is of interest to compare the values obtained with those reported in the literature for the CHT-A₄ system. A summary of the present results, together with corresponding values for CHT-A₄, is presented in Table V.

The first five constants listed show the two chymotrypsins to be quite similar, and even where larger variations are found, such as between the inhibition rates of DFP and DPC, the values remain within the same order of magnitude. As might be expected from previous studies and the general structural similarity of the two zymogens noted in the Introduction, the two chymotrypsins are very similar enzymically. The lower reaction rates of CHT-B with DPC and DFP, like the difference between deacylation rates of the acyl-enzymes, cannot be explained at the present time and, in the absence of further data, must remain as observations. It is rather interesting to note that anisoyl-CHT-B is less stable than the corresponding anisoyl-CHT-A₄, and this relationship is preserved for both the trimethylacetyl and cinnamoyl enzymes.

The phenomenon noted by Caplow and Jencks (60) of increased deacylation rates with A-CHT-A₄ in Tris buffer was also observed with A-CHT-B, but the rate increase was only twofold as opposed to 3.7-fold found by the above authors. A similar increase in hydrolysis rate of A-I in Tris was also found. Caplow and Jencks suggest the rate increase is due to acylation of the Tris, although no proof exists of this.

In conclusion, the results from this chapter show that CHT-B and CHT-A₄ have very similar enzymic properties. The

search for an ideal non-reversible inhibitor was only partly successful, but A-I was found to be suitable for use in end-group analysis, while DFP might be used as a more general inhibitor. Indole was demonstrated to be a reasonably efficient competitive inhibitor of CHT-B and can be used in activation experiments for limiting chymotryptic autolysis.

III. FACTORS AFFECTING THE ACTIVATION OF CHYMOTRYPSINOGEN-B

Chymotrypsin-B may be prepared from the zymogen under a variety of conditions. Thus the rate of activation, the maximum activity achieved, and the stability of the product are dependent upon such variables as the trypsin concentration, pH, ionic strength, specific ions, inhibitors, and temperature. One of the objects of the present research was to correlate the chemical and physical changes occurring in the zymogen molecule during activation with the levels of enzymic activity achieved. For this purpose, it was first of all desirable to study the generation of enzymic activity as a function of the variables listed above.

In general, the studies have been restricted to those conditions known to produce a rapid and maximal activation for the CHTG-A system, since the chemical events taking place in this system are discrete and well-documented (42,43). However, a few experiments have been done to demonstrate that slow activation of CHTG-B does occur and that the resulting level of activity is considerably lower than that for rapid activation.

A rapid activation scheme for CHTG-A was first proposed by Jacobsen (40), and the system was investigated more fully by Bettelheim and Neurath (42). These experiments showed that at pH 7.8 and 2°, a 2% solution of CHTG-A could be activated with a trypsin concentration of 0.07% (CHTG-A: trypsin ratio of 29:1) to a level 1.4 times as high as that

for CHT-A₄ (i.e. $k' = 4.6$) in approximately 30 minutes. When the activation was carried out in the presence of 0.1 M β -phenylpropionate, this competitive chymotryptic inhibitor was demonstrated to have little effect upon either the rate of activation or the esterase activity produced.

Enenkel and Smillie showed in a preliminary investigation that CHT-B gave similar activities to CHT-A₄ (15). However, in 1964 Krehbiel et al. (16) reported studies on the rapid activation of CHT-B at 37° which were not in complete agreement with this finding. The system employed was unusual in that the zymogen concentration was very low (0.02%), the trypsin:zymogen ratio rather high at 1 mole trypsin per 10 moles of zymogen, and all experiments were performed at 37° and pH 7.4, making comparison of the results with previously published data on the CHTG-A system difficult. The conclusions reached by these workers are quite interesting. They found that CHTG-B was activated more rapidly than CHTG-A, and over a wide range of trypsin concentrations, the generated CHT-B activity was approximately twice that of CHT-A. This latter result is somewhat surprising in view of all previously reported values for activities of CHT-A and CHT-B. In the experiments presented below, it will be shown that the second conclusion of these workers is invalid, and possible reasons for the result will be examined.

1. Effect of Ionic Strength and CaCl₂ on the Esterase Activity and Stability of CHT-B

Green et al. (46) examined the effects of Ca⁺⁺ ions upon the esterase activity of CHT-A₄, and Wu and Laskowski (47)

carried out a similar investigation on both CHT-A₄ and CHT-B. It is known that Ca⁺⁺ ions have a dramatic effect upon the stability and esterase activity of trypsin (46,91), but that this ion has much less influence on CHT-A₄. The results with CHT-B (47) indicate that Ca⁺⁺ has a variable effect upon the hydrolysis of protein substrates, while no data are available for ATEE hydrolysis. This prompted a review of the effects of both Ca⁺⁺ and ionic strength upon the CHT-B system.

a. Method

(i) Measurement of Esterase Activity

Chymotryptic activity was measured against 10 mM ATEE at pH 8.0 and 25^o, the substrate solution containing 0.01 M Tris-HCl and varying amounts of KCl or CaCl₂ according to the particular experiment. Hydrolysis of the substrate was followed in the pH-stat as described in Chapter II.1.

(ii) Activation of CHTG-B and CHTG-A

To a 1.0 ml volume of 1% zymogen at 0^o, in 0.1 M Tris-HCl, pH 8.0, containing CaCl₂ or KCl in appropriate experiments, was added 25 μl of 1% trypsin solution in 10⁻³ M HCl (CHTG:trypsin ratio, 40:1). At suitable times, 100 μl samples were removed and diluted in 5.0 ml 10⁻³ M HCl. Small volumes, 25 μl, could then be assayed in the pH-stat against a standard substrate solution consisting of 10 mM ATEE, 0.01 M Tris-HCl, 0.05 M CaCl₂, and 0.1 M KCl. Protein concentration was found by measuring A₂₈₀ as described in Chapter II. The dilute samples in 10⁻³ M HCl were stable at 5^o for at

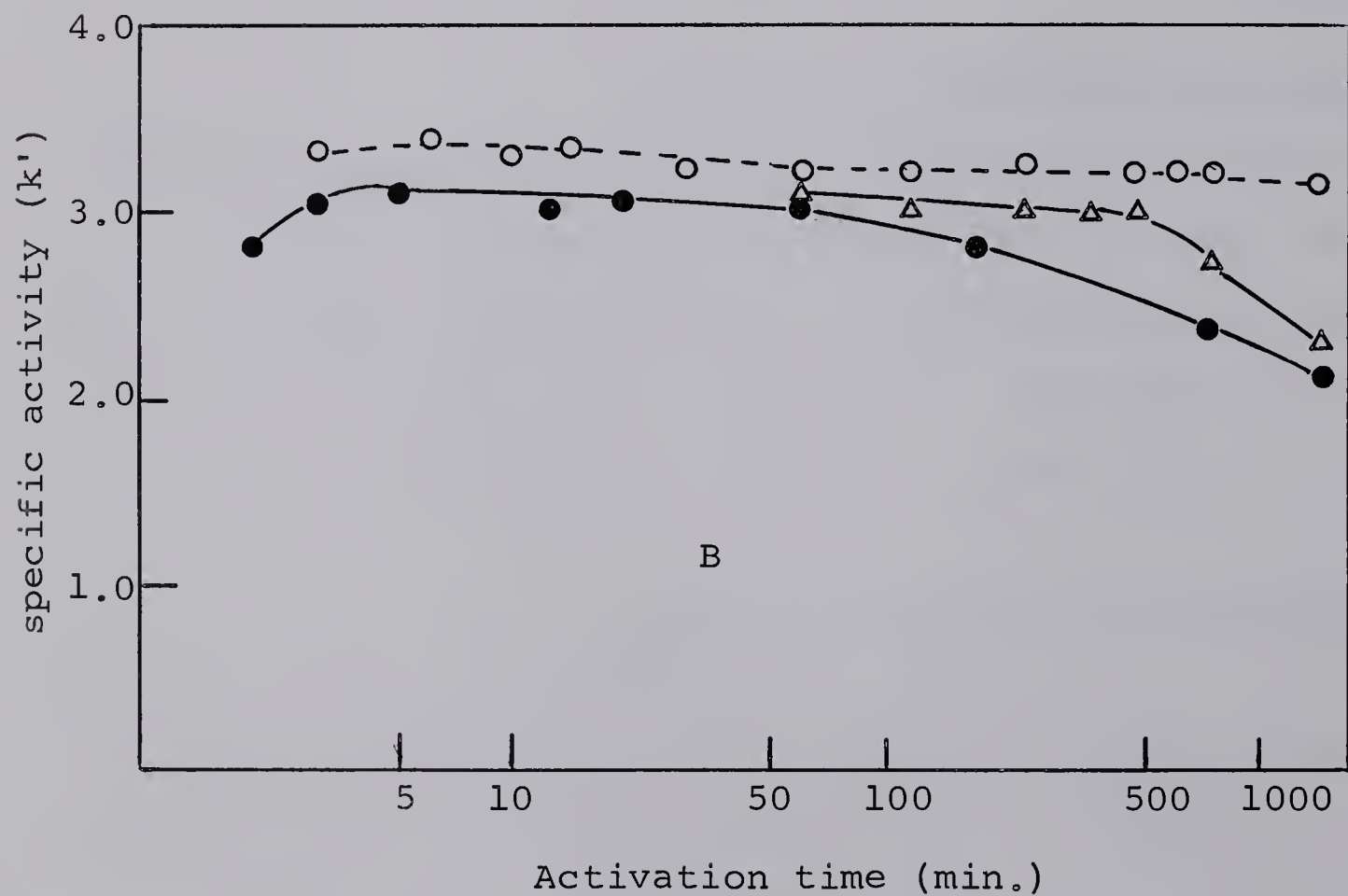
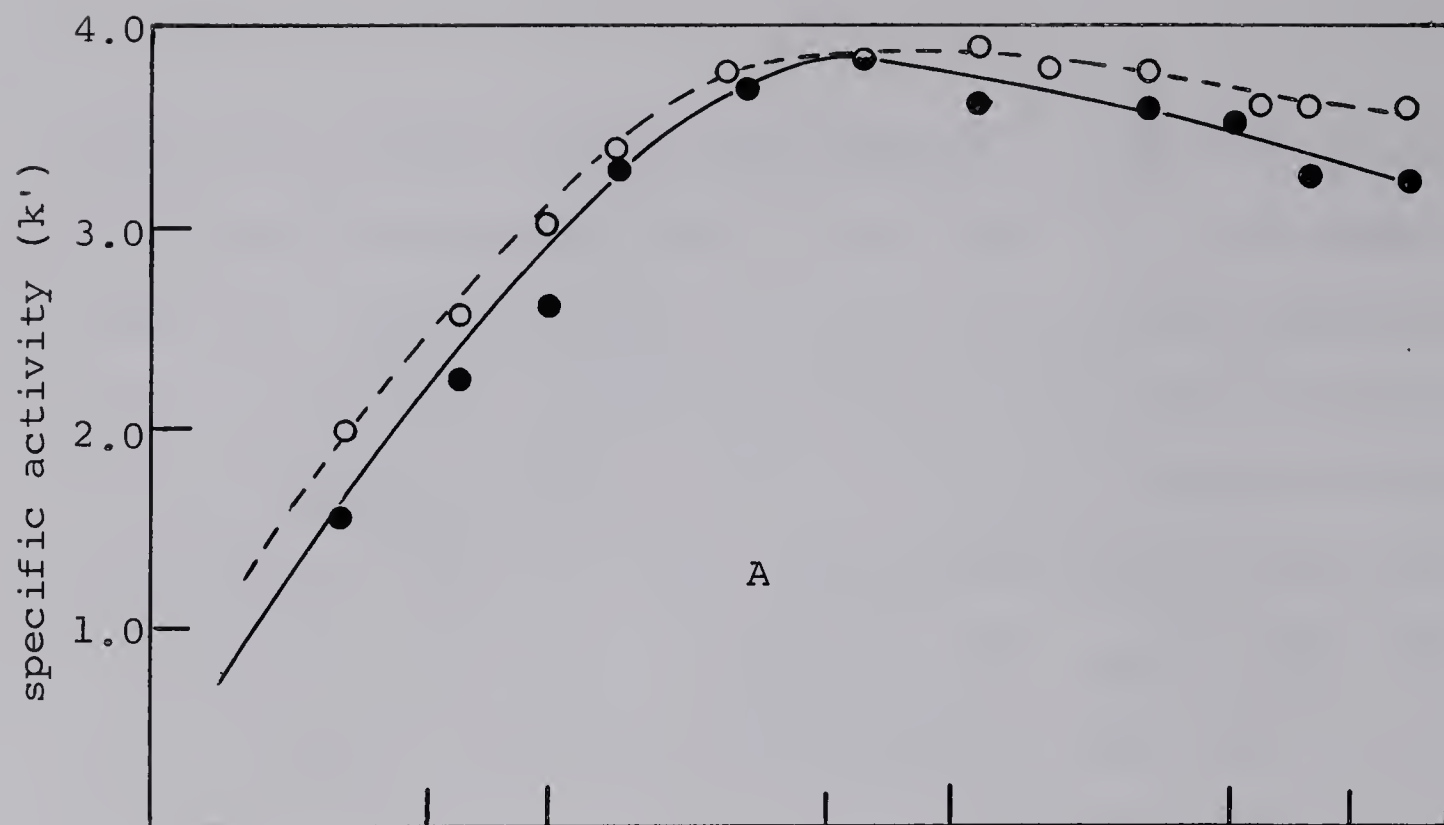


Figure 4. Effect of Ca^{++} ions and ionic strength on the activation of CHTG-A and CHTG-B and stability of CHT-A and CHT-B.

A CHTG-A in 0.1 M Tris-HCl, pH 8, 0°, 4×10^{-4} M CHTG-A 1×10^{-5} M trypsin.

B CHTG-B in 0.1 M Tris-HCl, pH 8, 0°, 4×10^{-4} M CHTG-B 1×10^{-5} M trypsin.

-○- 0.05M CaCl_2 ; -●- Tris-HCl alone; -△- 0.2M KCl.

Table VI
Influence of Ca^{++} and Ionic Strength on the
Esterase Activity of CHT-B₂

Additional Salt in Assay Solution	% Esterase Activity
0	100
0.01 <u>M</u> KCl	104
0.05 <u>M</u> KCl	112
0.20 <u>M</u> KCl	116
0.01 <u>M</u> CaCl_2	132
0.05 <u>M</u> CaCl_2	140
0.1 <u>M</u> CaCl_2	140

least 3 days.

b. Results and Discussion

The three major pancreatic endopeptidases, trypsin, CHT-A, and CHT-B, all respond to Ca^{++} ions in an apparently similar fashion. Trypsin esterase activity is increased 25% by the inclusion of Ca^{++} ions in the assay solution, and CHT-A₄ also shows more activity (46). Tests on CHT-B (Table VI) showed that both Ca^{++} ions and total ionic strength exert a strong influence on the hydrolysis of ATEE by the enzyme. An ionic strength of 0.2 results in a 15% increase in hydrolysis rate, while 0.05 M CaCl_2 produces a 40% rate increase. In view of these findings, routine esterase assays were all carried out on solutions of ATEE in the presence of 0.1 M KCl and 0.05 M CaCl_2 .

Little difference in the activation rate of either CHTG-A or CHTG-B could be demonstrated after the addition of Ca^{++} ions to the system, as illustrated in Figure 4. This result is in sharp contrast to the effect of Ca^{++} ions on trypsin activation. In the presence of this ion, full tryptic activity is generated, but in its absence the trypsinogen is converted to "inert protein" (97). All three enzymes, CHT-B, CHT-A, and trypsin, when left in solution at pH 7-8 for a long time, lose varying amounts of esterase activity (Figure 4 and reference 91). Nord and Bier (92) examined the stability of trypsin, and found that after 20 hours at pH 7.5 in the absence of Ca^{++} ions, the enzyme was 96% inactivated and much of the material remaining was dialysable, but only 50% inactivation occurred in the presence of Ca^{++} ions. While both

CHT-A and CHT-B are much more stable, the observed loss of activity upon prolonged incubation at pH 8 and 0° is prevented by Ca^{++} ions, and KCl also has some stabilising effect on CHT-B when present at high concentrations.

Kunitz and Northrop (96) suggested that trypsin exists in two forms, native and denatured, which are in equilibrium. This idea was used by Nord and Bier (91) to explain the effect of Ca^{++} ions on trypsin. The ions shift the equilibrium towards the native form by combining with it. As this form is more stable to proteolytic attack, loss of activity is prevented. The esterase activity may also be increased if these ions maintain the enzyme in a favourable conformation for interaction with substrate. The effect of Ca^{++} ions on CHT-B could be via a similar mechanism.

2. Effect of Trypsin Concentration on the Activation of CHTG-B

In the early work of Kunitz on pancreatic proteases, CHT-A₄ was prepared by activation with very small amounts of trypsin over a period of 3 days, while the fast activation system first described by Jacobsen (40) gave maximum activity in 30 minutes. Not only was the rate of activation increased, but the esterase activity produced by the latter system was 40% higher than that generated by the former. The only major difference between the two systems was an increase in the amount of trypsin used, i.e., the ratio of zymogen:trypsin was decreased from 10^4 :1 to 30:1. Clearly, then, the amount of trypsin used to activate a zymogen affects not only the rate of activation, but also the amount of activity produced.

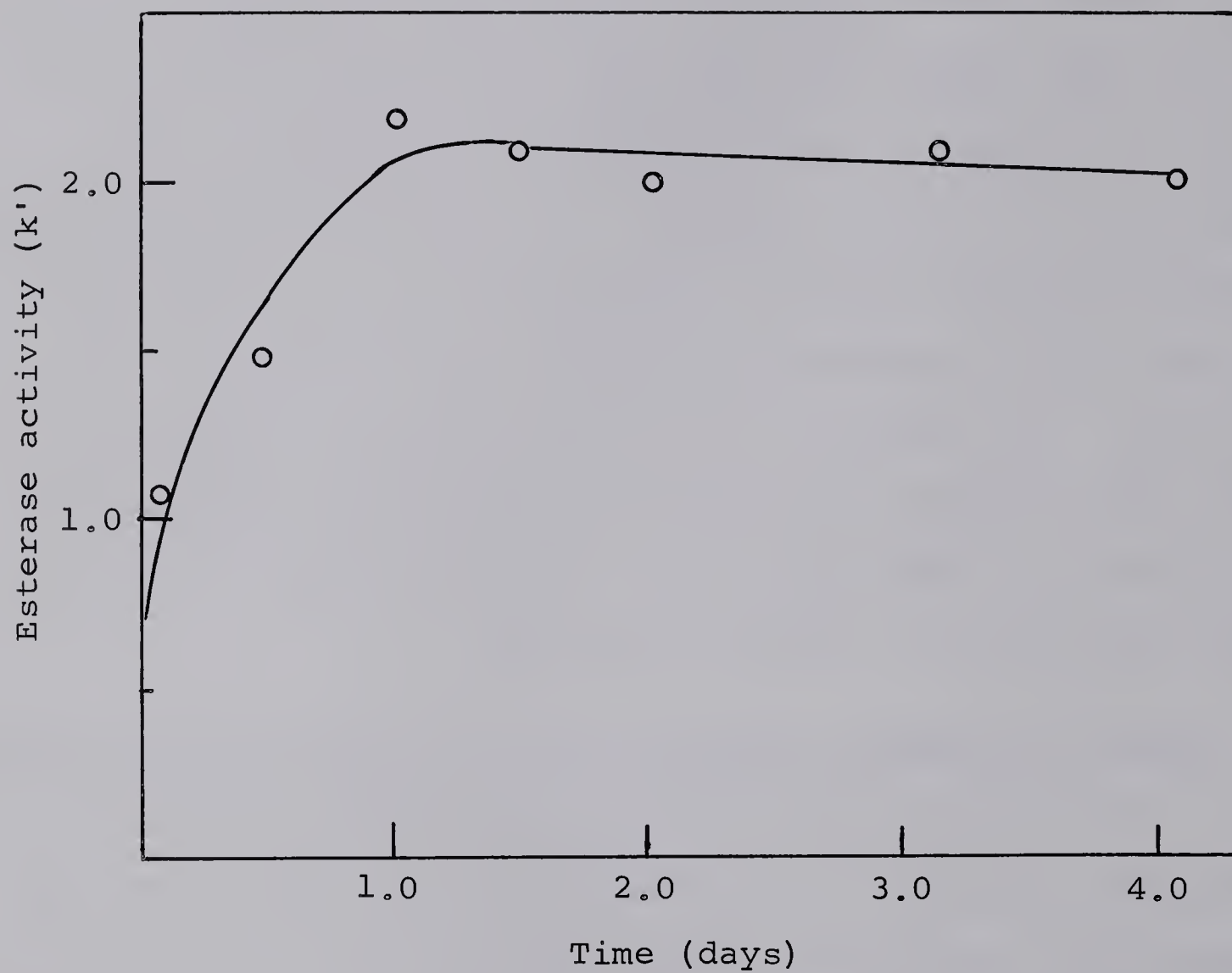


Figure 5. Slow activation of CHTG-B.

0.1 M Tris-HCl, pH 8.0; 0°, 4×10^{-4} M CHTG-B.

Trypsin = 1×10^{-7} M, Trypsin:CHTG-B ratio = 2.5×10^{-4} .

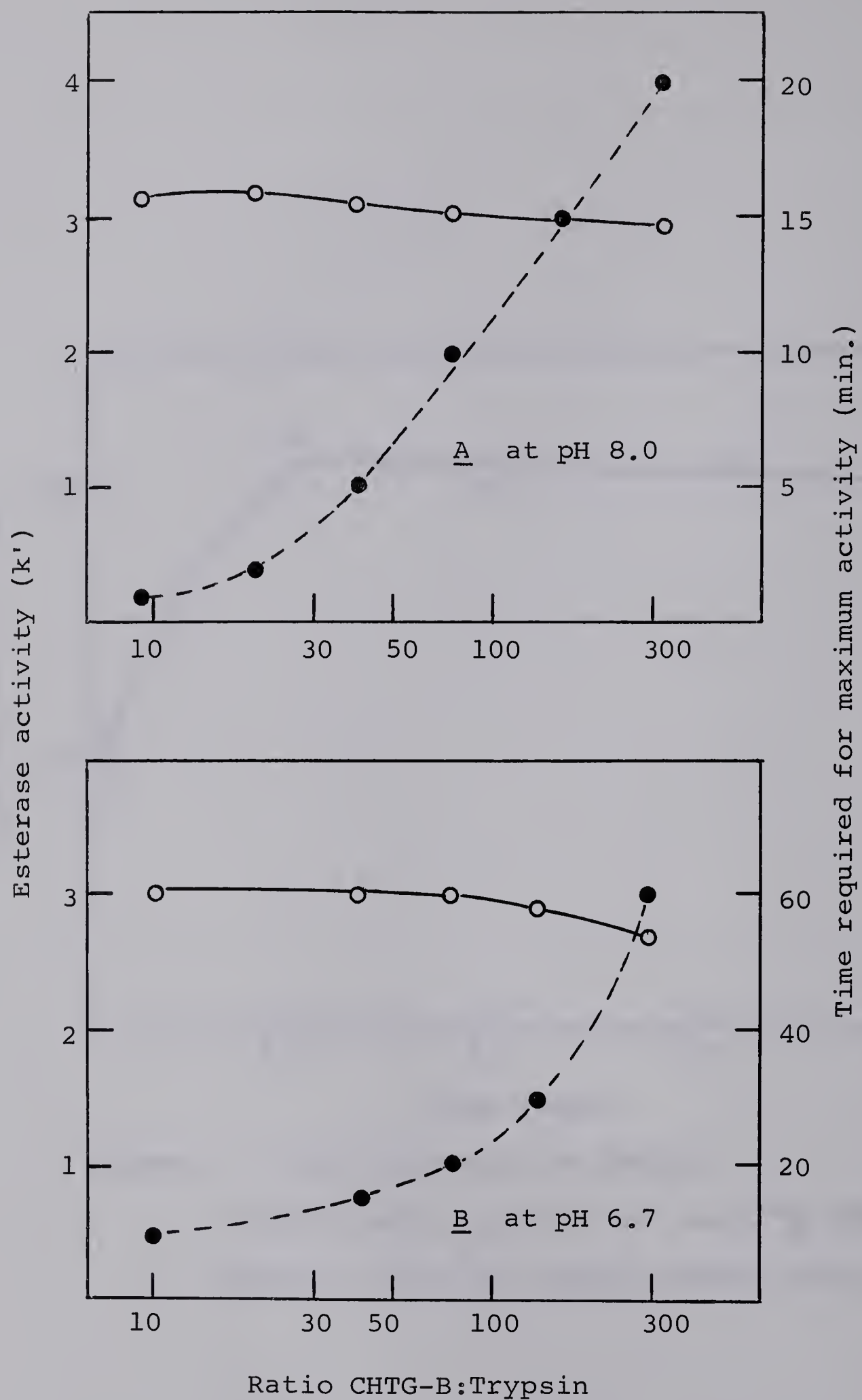


Figure 6. The effect of trypsin concentration on activation of CHTG-B.

-o- k'

-●- Activation time

Within the range of zymogen:trypsin ratios normally expected to produce rapid activation, there may also be variation in the above parameters. This series of experiments was designed to find the best concentration of trypsin for the generation of maximum CHT-B activity.

a. Method

Activation of a 1% solution of CHTG-B was carried out at 0° in either 0.1 M Tris-HCl buffer at pH 8.0 or 0.01 M phosphate buffer at pH 6.7. To 1.0 ml of zymogen was added 25 μ l of trypsin in 10^{-3} M HCl to produce the desired zymogen:trypsin ratio. Samples, 100 μ l each, were removed from the activation mixture at time intervals and quenched by dilution in 10^{-3} M HCl as previously described, before assay against ATEE. This method was used for all experiments on the rapid activation system and for the experiment simulating slow activation, although in the latter case, the concentration of trypsin was much lower.

b. Results and Discussion

Although the time required for full activation is dependent upon trypsin concentration, Figure 5 shows that the maximum esterase activity produced is not markedly influenced by variations in the concentration of this enzyme with ratios up to 100:1 of CHTG-B to trypsin. The most desirable method of preparing CHT-B would combine short activation time, high esterase activity production and minimal contamination with trypsin. A zymogen:trypsin ratio of 40:1 was chosen for all subsequent activations at both pH 8 and pH 6.7, as it was a

reasonable compromise fulfilling the above conditions. A very low trypsin concentration (slow activation) resulted in a much less active CHT-B preparation, probably because the CHT-B produced attacked the zymogen molecules to give neo-chymotrypsinogens that were not fully activatable. It is of interest to note that slow activation of CHTG-B results in an enzyme with comparable esterase activity ($k' = 2$) to that of a commercial preparation (Wilson Laboratories) produced by a similar method.

3. Effect of Indole upon the Activation of CHTG-B

In Chapter II, a series of competitive inhibitors was tested for the effect of each upon CHT-B and it was concluded that indole was the most suitable reagent. It was postulated that the inclusion of indole in an activation mixture would reduce chymotryptic autolysis and thereby exert some effect both upon the maximal esterase activity generated and the rate of its subsequent autolysis.

a. Method

Measurements of protein concentration by A_{280} were complicated by the high absorbance of indole in this region. The A_{280} of the zymogen was therefore measured in the absence of indole by preparing a 2% solution of CHTG-B in buffer and a 0.02 M solution of indole in buffer. A small sample (50 μ l) of zymogen was diluted in 10^{-3} M HCl and used for concentration measurement; equal volumes of protein and indole solution were then mixed to give final concentrations of 1% protein and 0.01 M indole. Activation with a CHTG-B:trypsin

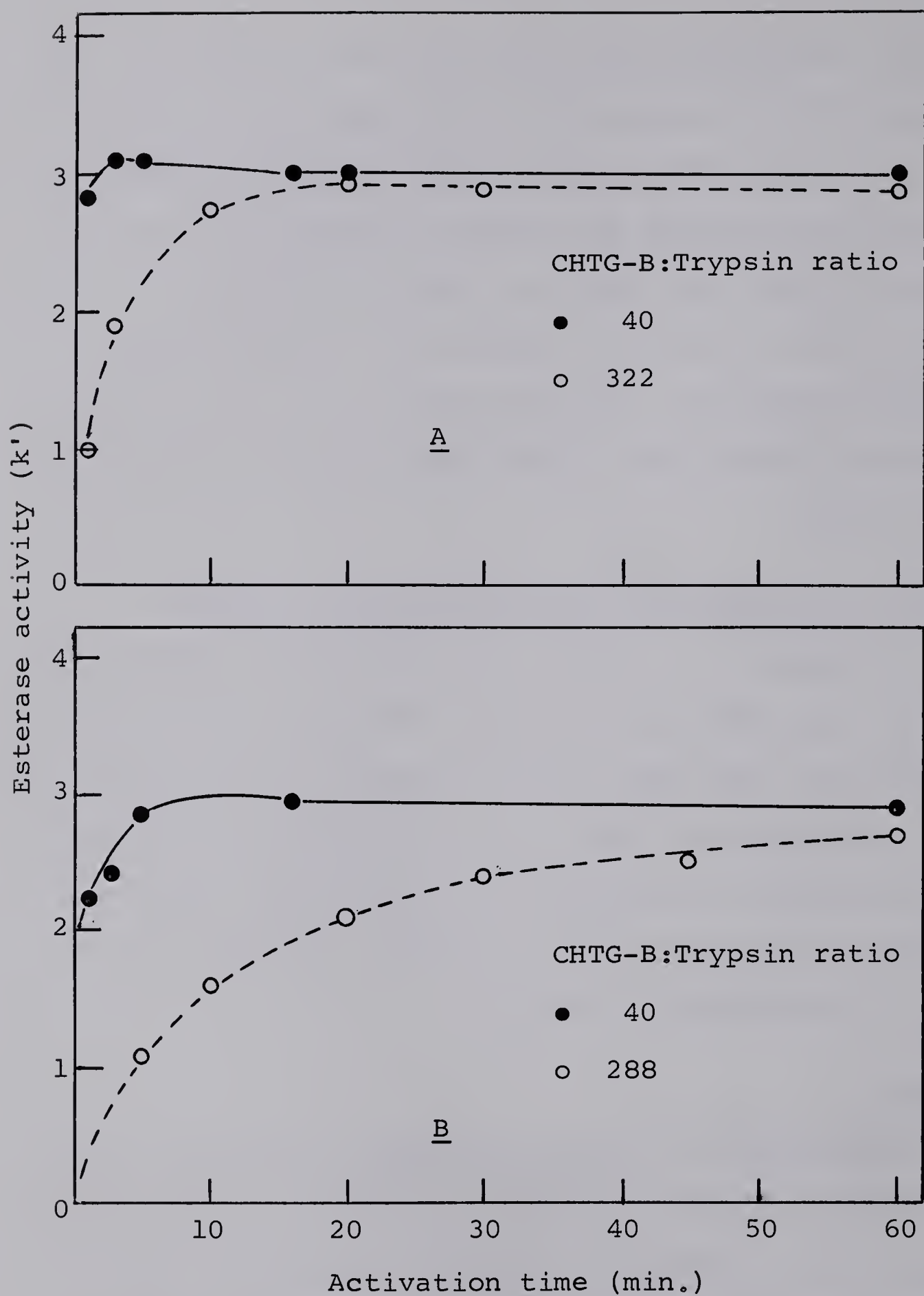


Figure 9. Effect of trypsin concentration on activation rate, at 0°C.

A at pH 8.0

B at pH 6.7

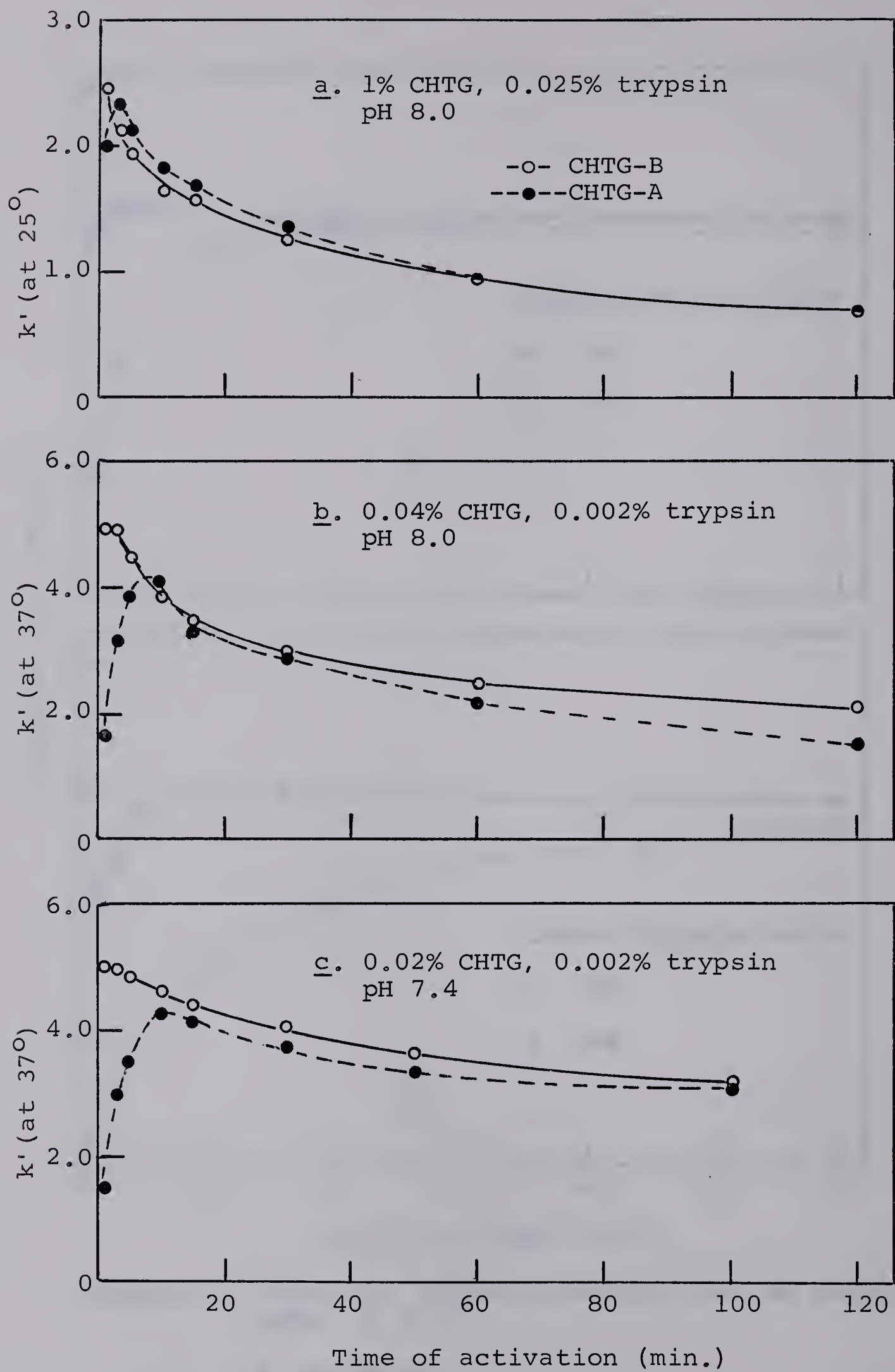
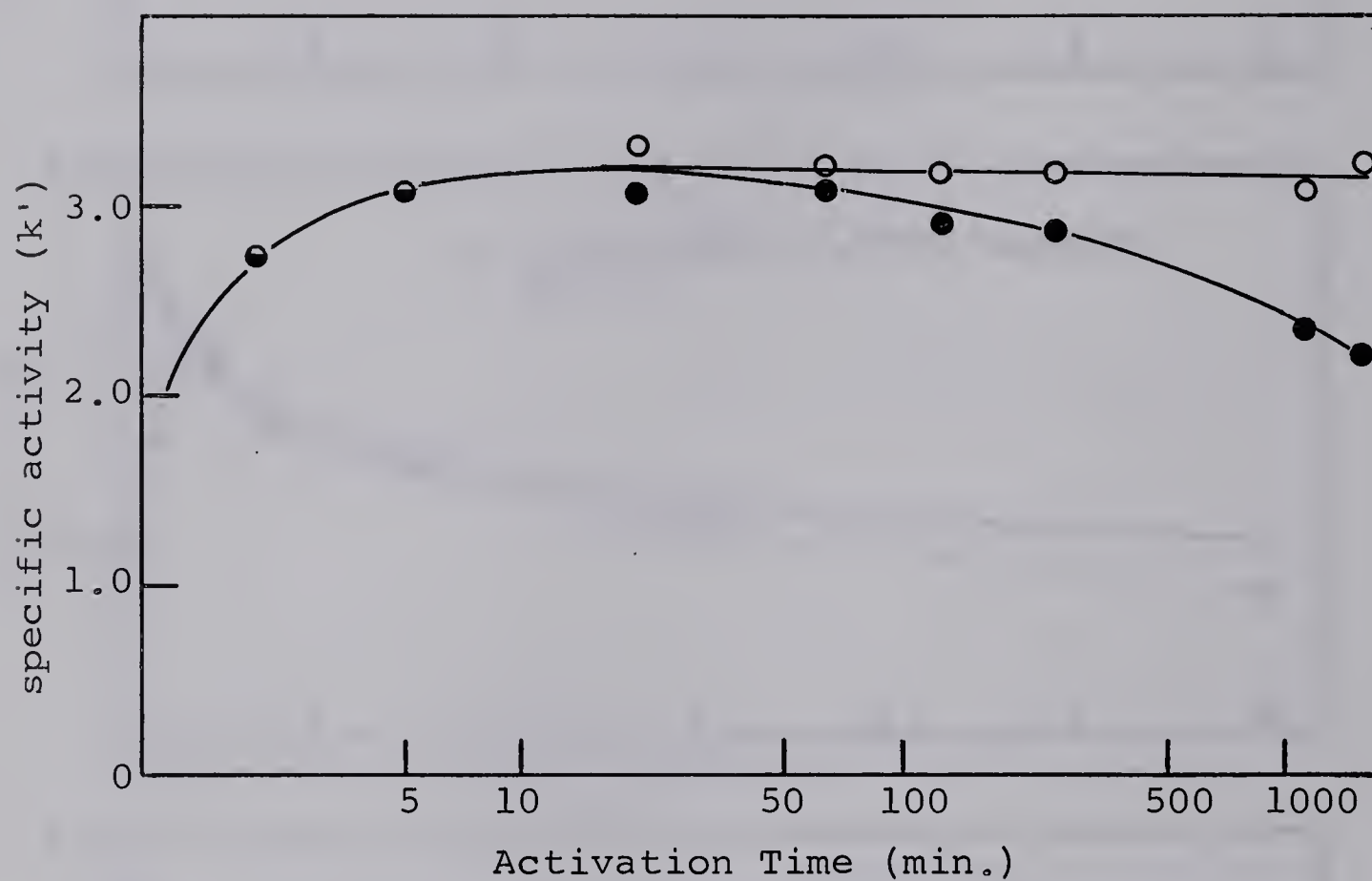


Figure 8. Activation of CHTG-A and CHTG-B at 37°.



—●— in 0.1 M Tris-HCl, pH 8.0, 0°

—○— in 0.1 M Tris-HCl, pH 8.0, 0.01 M indole, 0°

Figure 7. Effect of indole on CHTG-B activation and CHT-B stability.

ratio of 40:1 was then carried out followed by esterase determination of samples from the mixture, as previously described. The effect of indole upon the esterase activity during assay was considered to be negligible as the final concentration of the inhibitor was approximately 2×10^{-6} M.

b. Results and Discussion

Activation of CHT-B in the presence of indole follows a similar course to activation in the presence of CaCl_2 . The results in Figure 7 show that while the inhibitor has no effect upon the rate of activation or upon the esterase level produced, it maintains enzyme activity over a period of 24 hours with no noticeable loss. Despite the superficial resemblance of the actions of indole and Ca^{++} ions, indole acts primarily by inhibiting chymotryptic activity, although a change in enzyme conformation may accompany the formation of enzyme-inhibitor complex.

4. Effect of Temperature

Activation has been measured at two temperatures, 0° and 37° , under the usual conditions of 1% zymogen and a zymogen:trypsin ratio of 40:1 at pH 8.0. The results are reported in Figures 9 and 8a. As would be expected, a temperature increase accelerates both the activation and autolysis processes with the net result that a lower k' is produced and activity falls off more quickly at 37° than at 0° . Awad and Wilcox (29) obtained a similar result in their study of the activation of CHTG-A by Streptomyces griseus protease. At 40° , maximum activity was produced within 5 minutes and only

50% activity remained after 1 hour, while at 5° full activation required 90 minutes.

5. Effect of pH

The initial activation and subsequent autolytic processes are brought about by enzymes (trypsin and CHT-B, respectively) whose activities are a function of pH. It is therefore to be expected that the activation system will be pH-dependent. A comparison of the results in Figures 6 and 9 shows that both activation speed and maximum k' are reduced at pH 6.7 as compared with pH 8.0. The lower k' may arise as a result of the slower activation process in the same way as was suggested in part two of this chapter. The slow production of chymotryptic activity may allow the formation of neo-chymotrypsinogens by chymotryptic attack upon the zymogen prior to tryptic activation.

6. Comparison of CHTG-B Prepared in the Laboratory of Dr. M. Laskowski, and that of this Laboratory

The high esterase activity reported by Krehbiel et al. (16) raised an important question regarding the nature of the CHTG-B prepared in this laboratory, as attempts to duplicate these results, as shown in Figure 8c, were unsuccessful even with zymogen estimated to be 97% activatable. This question has been resolved by the generous gift from Dr. Laskowski of a sample of CHTG-B which was stated to be 90% as pure as the best preparation from that laboratory and contained 7% C-terminal tyrosine. Activation of this sample and a preparation of CHTG-B from this laboratory was carried out at pH 8.0

Table VII
Potential and Free Esterase Activity of
Two Samples of CHTG-B

Source of CHTG-B	Potential activity (k')		Free activity (k')
	5 min. Acti- vation	10 min. Acti- vation	
Dr. Laskowski's lab.	2.86	2.87	9.2×10^{-3}
Dr. Smillie's lab.	3.38	3.33	3.3×10^{-3}

and 0° , with a CHTG-B:trypsin ratio of 40:1. Esterase activity was measured against ATEE as described in part one of this chapter. The results, as given in Table VII, show the two preparations to be of similar activity, and on the basis of the observed k' of 2.86, the purest preparation from Laskowski's laboratory would have a k' of 3.2. (The relationship between k' and percent purity was described in Chapter I.)

In the work of Krehbiel et al. esterase activity is reported as μeq substrate hydrolysed per minute per μg enzyme after assay by the method of Peanasky and Szucs (93), at pH 7.0 and 37° ($k'_{\text{pH } 7.0, 37^{\circ}}$). The highest activity found by these authors was $k'_{\text{pH } 7.0, 37^{\circ}} = 1.2$, while a sample of CHTG-B prepared in this laboratory gave a $k'_{\text{pH } 7.0, 37^{\circ}}$ of 0.629 after activation and assay in the same manner. As the results of Table VII show the Laskowski material to be 97% as active as our own product, this latter activity may be corrected to $k'_{\text{pH } 7.0, 37^{\circ}} = 0.61$. It can be seen that the quoted figure is almost double our observed value, indicating that the discrepancy may be the result of arithmetical error. The quoted value for CHT-A activity ($k'_{\text{pH } 7.0, 37^{\circ}} = 0.58$) is in reasonable agreement with that observed in this laboratory ($k'_{\text{pH } 7.0, 37^{\circ}} = 0.53$). Dr. Laskowski (private communication) has agreed that such an error may exist and plans to re-investigate this question.

7. General Discussion

In the introduction to this chapter, three parameters

of an activation system were noted and their variation subsequently examined under various activation conditions in sections 1 to 5. The first of these parameters, activation rate, is influenced primarily by the rate at which trypsin attacks the zymogen molecule. This, in turn, is controlled by the total concentration of trypsin in the activation mixture and the temperature at which the activation is carried out, as an increase in either boosts the tryptic activity, and hence the rate at which the susceptible bond in the zymogen molecule is broken. If the rates of activation of CHTG-A and CHTG-B are compared, as in Figures 4 and 9, it is clear that CHT-B is generated much more quickly than CHT-A, a phenomenon that was also noted by Krehbiel et al. (16). While no simple explanation of this difference is available, it is noteworthy that Guy et al. (27) and Smillie (26) have demonstrated that position 14 in CHTG-B is occupied by an alanine residue. The corresponding residue in CHTG-A is serine. The former authors also note that during the activation of CHTG-B, the dipeptide Ala-Arg is not liberated, while in CHTG-A activation, the removal of Ser-Arg from an analogous position (see Figure 20) transforms CHT-A₁ into CHT-A₂. It may be suggested therefore that the substitution of an alanine for a serine residue in this critical position is responsible for the observed increase in activation rate.

The most active preparations of CHT-B result from activation at 0° with a relatively high concentration of trypsin. These conditions are equivalent to the rapid activation system, with trypsin to zymogen ratios of 1:10 or 1:100, devised by

Jacobsen. With smaller amounts of trypsin, i.e., slow activation, a lower k' is produced. Also, when rapid activation is carried out at 37° , an enzyme with lower esterase activity is formed. In both cases, the conditions allow greater chymotryptic autolysis to occur, with the result that the enzyme is greatly modified and loses activity. The suitability of the rapid activation system is emphasized by the observation that neither Ca^{++} ions nor indole produces a significant increase in esterase activity when activation is carried out in their presence.

When CHT-B is kept in solution at pH 8, the inevitable autolysis which takes place results in a loss of esterase activity. Temperature increases have a large effect simply by influencing the autolysis rate, as may be seen from a comparison of the results in Figures 8 and 9, where 60% of the activity is lost in 40 minutes (reckoned from 100% = k' of 3.36) at 37° , but a loss of only 30% occurs in 24 hours at 0° . An examination restricted to activation at 0° reveals that during both slow and rapid activation, two forms of enzyme appear to be stable. The first, produced by rapid activation, has a k' of approximately 3.3, while the other results from slow activation and has a k' of about 2. The stabilizing effect of indole points directly to the loss of activity being caused by chymotryptic autolysis. From these results, one may predict that there are at least two stable forms of CHT-B, a situation reminiscent of the CHTG-A system where CHT-A₂ and CHT-A₄ are produced by rapid and slow activation, respectively.

In conclusion, we have adopted the following system as the most suitable conditions for the maximal generation of chymotrypsin-B activity: 1% CHTG-B, 0.025% trypsin in 0.1 M Tris-HCl at pH 8.0 and 0° C. If desired, 0.01 M indole and 0.05 M CaCl₂ may be included in the activation system to prevent chymotryptic autolysis. In situations where less trypsin contamination is desirable in the final product, lower ratios of this activating enzyme may be employed with a longer activation time. The inclusion of indole and calcium is then important for that achievement of maximal activity.

IV. CHEMICAL CHANGES OCCURRING DURING THE ACTIVATION OF CHYMOTRYPSINOGEN-B

The previous chapters have described some of the enzymic properties of CHT-B and the effects of various conditions on the rate of activation, maximal activity achieved and the stability of that activity. Of the conditions studied, the combination chosen as most suitable was very similar to that previously employed by various workers (42,43) for their studies on the activation of CHTG-A. Thus a direct comparison of the enzymic, chemical and physical events involved in activation of the two zymogen systems is facilitated.

In Chapter III, it was shown that the rapid generation of chymotrypsin-B activity by trypsin is followed by a slow drop in activity which can be largely prevented by the inclusion of indole and calcium in the activation system. This would suggest that further autolytic chymotryptic splits are occurring in the CHT-B molecule, leading to a decreased enzymic efficiency of the molecule in a manner analogous to the well-documented conversion of CHT-A₁ and CHT-A₂ to CHT-A₄. Previously published work (16,27) on the CHT-B system, however, indicates that the chemical events are not the same as those occurring in chymotrypsin-A autolysis. For example, Guy et al. (27) have reported, without describing experimental details, that no specific peptides were released during activation of CHTG-B. They were able to isolate the A' chain of

CHT-B (after activation by trypsin in the presence of 0.1 M β -phenylpropionate for 1.5 hrs.) and show that not only was the bond between residues 13 and 14 intact, but that the serine-14 of CHTG-A is replaced by alanine in CHTG-B. This substitution was suggested as the reason for the lack of activation peptide. A similar negative result has been reported by Krehbiel et al. (16) but, again, no experimental details were described. The apparent lack of liberated peptides during activation does not mean, of course, that further autolytic cleavages do not occur since the polypeptide chains so formed could be held together by disulfide, hydrogen or hydrophobic bonds. Indeed, N- and C-terminal analyses by previous workers would indicate that such cleavages do take place. Thus it is quite possible that more than one form of active CHT-B is produced as a result of autolysis following the initial tryptic activation.

Because of the incomplete and unsatisfactory nature of the reported experimental findings bearing on the chemical changes during the activation of CHTG-B, it was decided to reinvestigate this question in some detail. For this purpose, we have carried out a search for peptides liberated during activation. In agreement with previous reports, no such peptides were detected in significant amounts. In addition, N- and C-terminal analyses have been made at various stages during the activation which indicate that at least one, and probably more, peptide bonds are susceptible to chymotryptic autolysis. This finding has been substantiated by the separation of two polypeptide chains from CHT-B by chromatography

on DEAE-cellulose in 8 M urea after reduction and carboxymethylation of the disulfide bonds.

Besides studies on the chymotrypsin-B prepared in this laboratory by activation of CHTG-B, we have also done some chemical studies on a preparation of chymotrypsin-B available commercially from Wilson Laboratories, Inc., Chicago (hereafter referred to as Wilson CHT-B). This material is prepared under conditions of slow activation as described by Laskowski (95) and was found to have a lower activity towards ATEE than the enzyme prepared in this laboratory. Although the k' was only 2.0, titration with C-I and A-I indicated that it was 85% homogeneous by this criterion. Column chromatography on CM-cellulose gave a single peak in the same position as chymotrypsin-B prepared in this laboratory (15). The studies described below indicate that it is a form of chymotrypsin-B in which extensive cleavage of at least two peptide bonds has occurred.

1. Examination of the Activation System for Liberated Peptides

The search for peptides liberated during activation was done in several ways. Initially, the amino acid composition of a trichloroacetic acid (TCA) supernatant of the activation mixture was examined for constituent amino acids after hydrolysis with HCl. Subsequently, the TCA supernatant was separated into low and high molecular weight fractions on Sephadex G-25 and the amino acid composition of the low molecular weight fraction examined. Finally, the peptide fraction from the TCA separation was examined by chromatography on a

Table VIII

Amino Acid Analysis of Small Peptide Fraction Produced
During Activation of CHTG-B. Expressed as
Moles Amino Acid per Mole of Protein Activated

Amino Acid	1 hr. Acti- vation and G-25 chrom.	2 hr. Acti- vation and TCA ppn.	8 hr. Acti- vation and TCA ppn.	24 hr. Acti- vation and TCA ppn.
Lys	0.86	0.59	0.81	0.89
His	0.15	0.15	0.17	-
Arg	0.52	0.37	0.34	0.37
Asp	0.97	0.75	0.91	1.29
Thr	0.63	0.44	0.58	0.86
Ser	0.81	0.67	0.82	1.22
Glu	0.92	0.55	0.57	0.83
Pro	0.61	0.49	0.50	0.75
Gly	0.78	0.55	0.77	1.23
Ala	0.91	0.71	0.89	1.54
Val	0.84	0.59	0.72	1.04
Met	0.14	0.05	0.05	1.09
Ile	0.42	0.27	0.34	0.47
Leu	0.82	0.60	0.82	1.31
Tyr	-	0.19	0.22	0.29
Phe	0.23	0.21	0.24	0.33

column of Dowex 50.

a. Methods

(i) Amino acid composition of TCA supernatant

Four μ moles of CHTG-B were activated under standard conditions (1% CHTG-B, 0.1 M Tris, pH 8.0, 0°C, ratio CHTG-B:trypsin, 1:40) for 24 hours. At 2 hours, 8 hours, and 24 hours, samples (1.0 μ mole in 2.5 ml of solution) were added to 2.5 ml of cold 30% TCA and centrifuged. After extraction of the TCA supernatant with peroxide-free ether, the aqueous solution was dried and hydrolysed with 6 N HCl in a sealed, evacuated tube at 105°C for 18 hours. The HCl was removed under reduced pressure at 50°C and the sample analysed on the 10 and 50 cm columns of the Beckman automatic amino acid analyser, Model 120B. The results, expressed as moles of amino acid liberated per mole of protein are given in Table VIII.

(ii) Amino acid composition of Sephadex G-25 low molecular weight fraction

Similar TCA soluble fractions from a 1 hour activation mixture were chromatographed on a column (150 x 1.1 cm) of Sephadex G-25 in 0.05 N acetic acid at 5°C. The TCA soluble equivalent of 1 μ mole of CHTG-B was extracted with ether and dried as before; it was then dissolved in 0.05 N acetic acid and applied to the column. A sample of ϵ -DNP-lysine was also applied to act as an indication of complete elution. The effluent was monitored by measuring A_{280} and by ninhydrin analysis (described in Appendix B). The column performance was checked by chromatographing a sample of CHTG-B and a

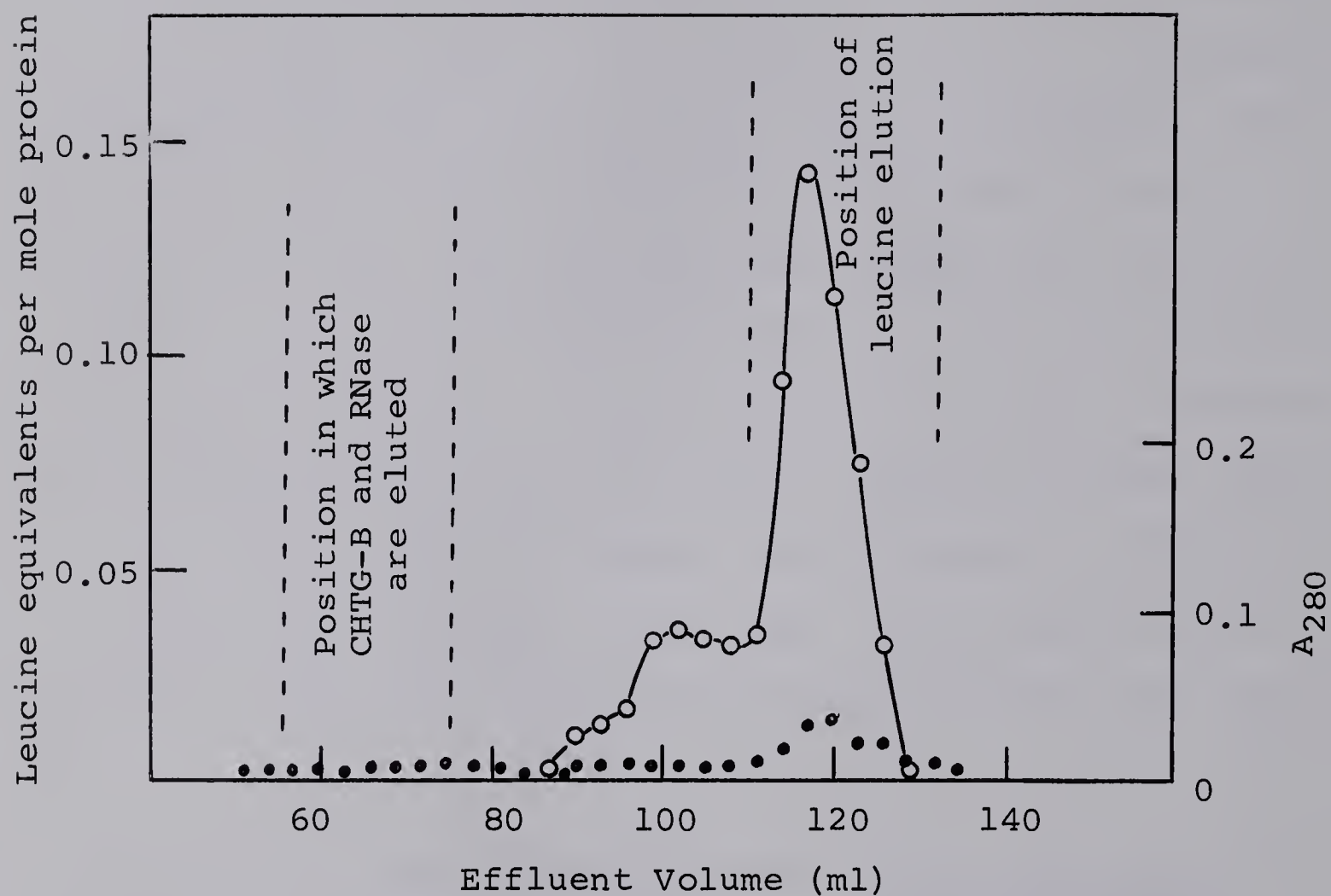


Figure 10. Chromatography of TCA soluble fraction from CHTG-B activation mixture on Sephadex G-25.

●●● Absorbance at 280 mμ.

-o- Ninhydrin colour.

ribonuclease-leucine mixture. The position at which each was eluted is shown on Figure 10. Low molecular weight ninhydrin positive samples were pooled, dried, and hydrolysed with 6 N HCl before amino acid analysis.

(iii) Fractionation of TCA-soluble material on Dowex-50

A column of Dowex 50 x 2 (0.9 cm x 100 cm) was prepared according to Schroeder et al. (71). The resin (150 g, 200-400 mesh, BioRad Laboratories) was washed on a Buchner funnel with 400 ml water, 450 ml N NaOH, 450 ml water, 300 ml 2 N HCl, 450 ml water, 400 ml 2 N pyridine and 300 ml of pH 3.1 buffer and suspended in twice its volume of pH 3.1 buffer, (16.1 ml of redistilled pyridine, 278.5 ml of glacial acetic acid made up to 1 liter).

The column was poured in a glass tube fitted with a water jacket and packed with slight air pressure. Before use, the temperature of the resin was raised to 38° by circulating water through the jacket, and equilibrated by passing 150 ml of pH 3.1 buffer through the column. A TCA soluble fraction prepared from 1 μ mole of CHTG-B activated for 1 hour and brought to pH 3.1 with 6 N HCl, was charged onto the column and allowed to flow in under gravity. The sample, washed in with two 1 ml volumes of pH 3.1 buffer, was eluted with a slightly convex gradient of pH and pyridine concentration. Three chambers of a Varigrad (Buchler Instruments, Inc.) were used to prepare the gradient and a constant flow rate was maintained with a peristaltic pump (Sigma Motor Inc., Model T8). Chamber 1 of the Varigrad contained 150 ml of 0.2 M

pyridine adjusted to pH 3.1 with acetic acid, chambers 2 and 3 each contained 150 ml of 2 M pyridine adjusted to pH 5.0 with acetic acid (161 ml pyridine, 143 ml glacial acetic acid per liter of buffer). A total of 220 fractions, each of 1.8 ml, were collected at the rate of 8 ml per hour before discontinuing the gradient and passing through 2 N NaOH to remove any basic material.

A portion (0.25 ml) from each sample was assayed after hydrolysis in 1.25 ml of 2.5 N NaOH for 2.5 hours at 95° (72). The hydrolysates were neutralized with 1.25 ml of 30% (v/v) acetic acid, 1.25 ml of ninhydrin solution added and the mixture heated for 20 minutes on a boiling water bath. The ninhydrin colour was measured at 570 mμ.

b. Results and Discussion

While both Guy et al. and Krehbiel et al. reported that no specific peptide was produced during activation, neither group specify how much peptide material was found. The experiments with TCA precipitation and Sephadex chromatography reveal that a considerable quantity of low molecular weight peptides are produced. Ninhydrin analysis of the Sephadex peptide fraction without hydrolysis indicates about 0.7 leucine equivalents per mole of activated zymogen, and the amino acid analyses in Table VIII give a total of 9.6 residues per mole of zymogen. Neither of these experiments, however, can indicate the number of peptides contributing to the soluble fraction, although the elution pattern from Sephadex G-25, Figure 10, gives an asymmetric peak which is almost split into

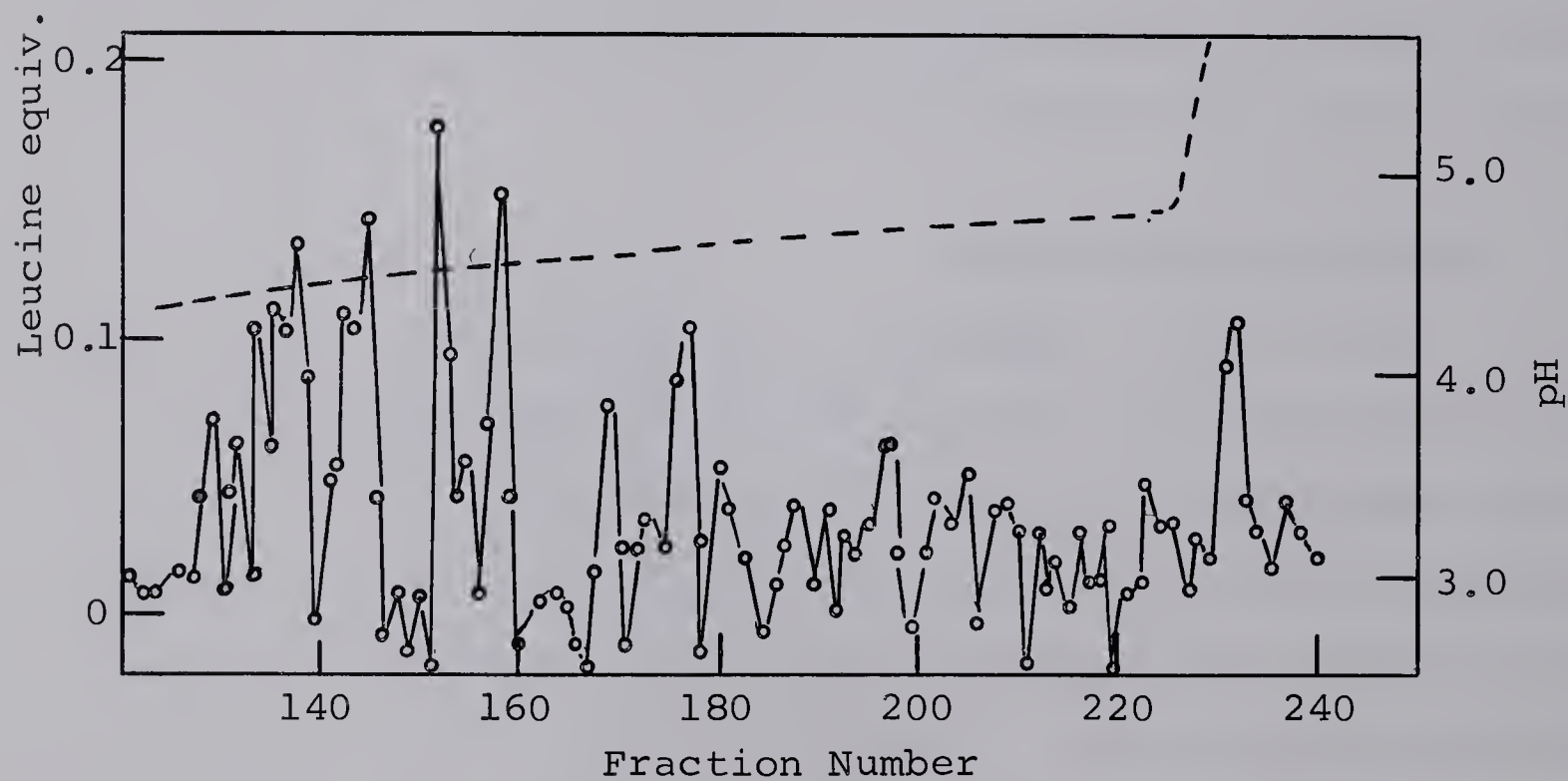
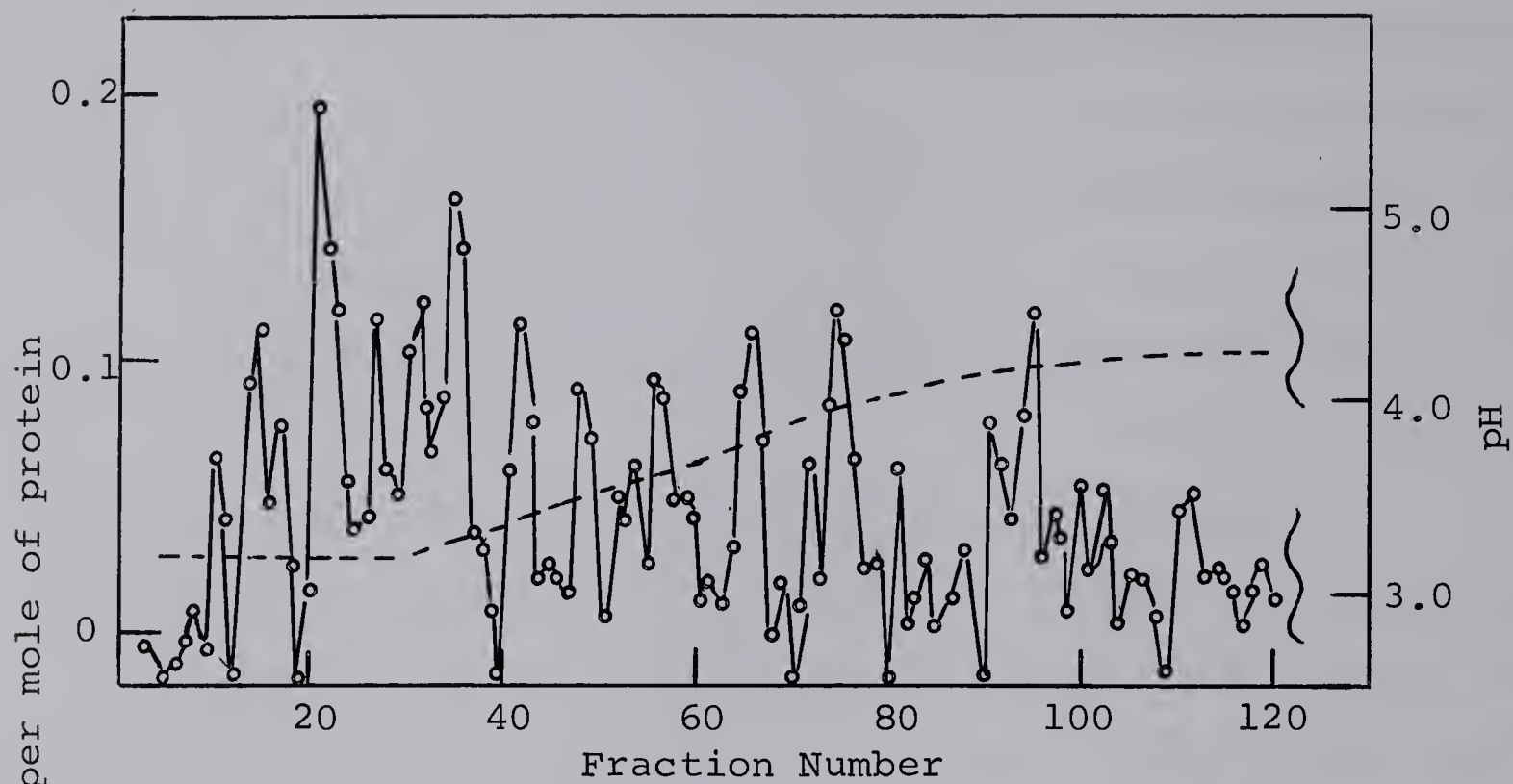


Figure 11. Fractionation of TCA soluble peptides from CHTG-B activation mixture on Dowex 50x2.

--- pH gradient

-o- ninhydrin colour

two parts, suggesting that the fraction is heterogeneous.

The question is resolved by chromatography on Dowex-50 as shown in Figure 11. The peptide fraction was separated into a large number of small peaks, each of which, even after hydrolysis, comprised less than 0.5 leucine equivalents per mole of protein. More specifically, no large amount of basic peptide was found which could correspond to the activation dipeptide Ala-Arg. A check of the total leucine equivalents eluted from the column indicated that all the peptide material had been accounted for. The total leucine equivalents eluted were 9.9, which compare quite closely to the value of 9.6 moles of amino acid per mole of protein found by amino acid analysis. The activation of CHTG-B is therefore different from CHTG-A in that no basic dipeptide is produced in anything close to molar quantities. However, a considerable amount of peptide material is formed in non-molar quantities, presumably arising from autolytic non-specific cleavages.

2. N-terminal Analyses

The chemical changes occurring during activation of chymotrypsinogen-B were further investigated by N-terminal analysis. Of the several available methods for the determination of N-terminal residues in proteins and peptides, the dinitrophenylation technique of Sanger has many advantages, including the relatively small amount of material required, simplicity of technique and apparatus and easily detectable coloured derivatives. It has been successfully employed with many proteins. Detection of new end groups formed during

activation is simplified in the CHTG-B system because, like CHTG-A, CHTG-B has an N-terminal half cystine (28) which may only be detected after conversion to cysteic acid by performic acid oxidation. Any 2,4 dinitrophenyl-amino-acids (DNP-amino acids) are therefore the result of peptide bond cleavages associated with activation or autolysis.

a. Method

Samples of CHTG-B were activated at 0° , pH 8.0 with a zymogen:trypsin ratio of 40:1 for 5 minutes or 60 minutes. After activation, the solutions were brought to pH 3.0 with 6 N HCl, dialysed exhaustively against 10^{-3} M HCl and freeze-dried. The freeze-dried protein was used for N-terminal analysis.

The procedure for dinitrophenylation, identification, and quantitative estimation of DNP-amino acids was modified from Fraenkel-Conrat et al. (74). While dinitrophenylation in concentrated urea (i.e. addition of pure 1 fluoro-2,4-dinitrobenzene (FDNB) to a solution of protein in 8 M urea maintained at pH 8.0 in the pH stat at 40°) works satisfactorily with many proteins, low and variable yields of DNP-amino acids were encountered when CHT-B samples were treated in this way. Various organic solvents were added to the dinitrophenylation system in an attempt to improve the yield of DNP-amino acids. It was found that 40% v/v dimethylformamide (DMF) did not precipitate the protein and brought the FDNB into solution, allowing a significant increase in reaction rate. The conditions finally adopted for dinitro-

phenylation were 60% 8 M urea, buffered at pH 8.5 with 0.1 M Tris, and 40% DMF at 25^O, for 2 hours. Tests with ribonuclease and CHT-A₄ showed that this system was effective (see Appendix D). It was also noticed that DNP-CHT-B was not readily soluble in 6 N HCl, but that it could be dissolved in 97% formic acid (Eastman Kodak) and the addition of an equal volume of 6 N HCl did not precipitate the protein. Hydrolysis of the DNP-enzyme was therefore performed in an equal mixture of 97% formic acid and 6 N HCl.

Standard preparations of DNP-amino acids (Mann Research Laboratories) were tested for destruction and losses during hydrolysis and extraction in the presence of an equimolar amount of DNP-CHTG-B. The recoveries of ether-soluble-DNP-amino acids reported in Appendix C were used to correct results from subsequent experiments on CHT-B.

Although most of the DNP-amino acids tested were recovered in high yield from both chromatography (96-98%) and after ether extraction from dilute HCl followed by chromatography (84-94%), bis-DNP-lysine showed considerable losses. After chromatography, 14% was lost and the recovery fell to 72% after both ether extraction and chromatography. Bis-DNP-lysine was also the most labile derivative under the conditions of hydrolysis. Only 61% was recovered after 12 hours, and 54% after 18 hours. In comparison, 90% of DNP-isoleucine, 72% of DNP-alanine, and 74% of DNP-aspartic acid were recovered after 12 hours and after 18 hours the recoveries were 86%, 64%, and 66%, respectively.

A typical N-terminal analysis by this technique was carried out as follows. Forty mg of protein, dissolved in 5.0 ml of 8 M urea, pH 3.0, containing 10 μ l of 1 M DFP, was brought to pH 8.5 by the addition of solid Tris. DMF (4 ml) was mixed into the solution, followed by 100 μ l of FDNB (Eastman Kodak) and the solution left in the dark at 25^o for 2 hours. DNP-protein was precipitated with 5 ml of 1 M HCl, washed twice with ether, 3 times with water, 3 times with acetone, twice with ether, and dried in vacuo over P₂O₅.

A known weight (about 6 mg) of DNP-protein was placed in an 18 x 200 mm pyrex tube and dissolved in 0.5 ml of 97% formic acid, before adding 0.5 ml of 6 N HCl. The end of the tube was cooled in dry-ice/acetone during evacuation to minimize bubbling. After evacuation, the tubes were sealed and kept at 105^o for 12 or 18 hours for hydrolysis. The hydrolysis tubes were cooled, opened, and the contents diluted with 5 ml of distilled water. DNP-amino acids were extracted with four 5 ml portions of peroxide free ether. The ether solutions were dried on a rotary Evapo-Mix (Buchler Instruments) and the residue taken up in a small volume of acetone for chromatography. Preliminary tests on the aqueous residue indicated that it contained only ϵ -DNP-lysine and im-DNP-histidine. Further work was therefore confined to the ether-soluble derivatives.

Separation was carried out by two-dimensional chromatography on Whatman No. 4 paper, which had been dried after dipping in 0.05 M phthalate buffer, pH 6.0. Papers were

equilibrated in the tanks with tert-amyl-alcohol-saturated water prior to separation in the first dimension by the system of Blackburn and Lowther using descending chromatography with tert-amyl alcohol saturated with phthalate buffer. For the second dimension, the papers were dried, turned through 90° and subjected to descending chromatography with 1.5 M phosphate buffer, pH 6.0.

DNP-amino acids were identified by comparison with standard mixtures and estimated quantitatively after elution from the paper with 1% sodium bicarbonate solution. The coloured spots, together with appropriate blanks were excised, placed in separate test tubes and 5.0 ml of sodium bicarbonate solution added to each. After 1 hour at 25° the solutions were centrifuged to remove debris, and the absorbance read at 360 m μ .

To confirm the identification, standard DNP-amino acids and those derived from CHT-B and CHT-A₄ were separated by thin layer chromatography on silica Gel G (Welch Scientific Co. Ltd.) by the method of Drawert et al. (75). Glass plates, 200 x 200 mm were coated with gel to a thickness of approximately 0.2 mm and mixtures of DNP-amino acids applied at one corner. Ascending chromatography was performed in the following solvents.

Dimension 1. Toluene; pyridine; 2-chloroethanol; 0.8 N NH₄OH (100:30:60:60). The lower, aqueous, phase was used for equilibration and the upper for development.

Dimension 2. Chloroform; benzylalcohol; glacial acetic acid (10:3:3).

Table IX

N-terminal Analysis of Chymotrypsin-B

Results expressed as moles of DNP-amino acid per mole of DNP-protein

DNP-protein	Hydrol. time	DNP-amino acid				
		Isoleucine* uncor. corr.	Lysine uncor. corr.	Aspartic Acid uncor. corr.	Alanine uncor. corr.	Threonine uncor. corr.
CHT-B 5 min. activation	16	0.67	0.09	0.03	0.04	Trace
	24	0.66	0.09	0.02	0.04	Trace
	Average	0.81	0.16	0.04		Trace
CHT-B 60 min. activation	16	0.67	0.41	0.03	0.05	0.10
	24	0.64	0.41	0.04	0.08	0.06
	Average	0.77	0.71	0.06	0.12	0.15
Wilson CHT-B	18	0.61	0.53	Trace	0.08	0.13
	24	0.60	0.50	Trace	0.10	0.18
	Average	0.71	1.00	Trace	0.15	

* Sum of DNP-Ileu and DNP-Ileu-Val.

b. Results and Discussion

For this examination of the N-terminal groups, three types of chymotrypsin-B were used, two of which had been produced by rapid activation of CHTG-B at pH 8.0 for differing times, while the third was Wilson CHT-B. The results (Table IX) show that the only major difference between enzyme activated for short or long periods is the appearance of N-terminal lysine. This residue is also found, in higher yield, in CHT-B prepared by slow activation. As with CHT-A, the isoleucine-valine bond is relatively resistant to acid hydrolysis so that both DNP-isoleucine and DNP-Ile-Val are found in the ether extracts. The value for N-terminal isoleucine reported in Table IX is the sum of both DNP-isoleucine and DNP-Ileu-Val spots. The molar extinction coefficient of DNP-isoleucine was used to calculate the quantity of DNP-Ileu-Val. This identification was confirmed by a comparison of the DNP-amino acid patterns from CHT-A₄ and CHT-B. Similarly bis-DNP-lysine from CHT-B chromatographed in the same position as standard bis-DNP-lysine, and the bis-DNP-lysine obtained from the N-terminal residue of ribonuclease.

Previous workers on this system have shown that isoleucine is the N-terminal group associated with the initial tryptic hydrolysis of CHTG-B, and the results on the enzyme produced by 5 minute activation confirm this. The presence of N-terminal lysine must therefore be attributed to chymotryptic autolysis, indicating that a second major split occurs in the zymogen molecule. In addition to N-terminal lysine, DNP-aspartic acid, alanine and threonine were found in low

yields. These may arise by autolysis either from minor cleavages in the polypeptide chain or from the production of small peptides in non-molar quantities. As will be demonstrated subsequently, however, the quantities and number of N-terminal residues observed are less than those from C-terminal analysis. This discrepancy may arise in part from the fact that in the N-terminal analyses, the DNP-protein is recovered from the reaction medium by precipitation with hydrochloric acid. Thus dinitrophenylated low molecular weight peptides would remain in the supernatant and be selectively removed during the preparation of the DNP-enzyme. Such DNP-derivatives would therefore not appear in the final analyses.

3. C-terminal Analysis

N-terminal analyses may be performed by a number of well-known chemical techniques, but no such generally applicable methods have been devised for C-terminal analysis. Instead, two exopeptidase enzymes have been isolated and used for this purpose. Carboxypeptidase-A (CP-A) will remove C-terminal amino acids provided a basic residue or proline is not terminal and proline is not sub-terminal. It has a high specificity for C-terminal aromatic and aliphatic hydrophobic residues, but is less effective for aliphatic polar and acidic amino acids (55). Carboxypeptidase-B removes only C-terminal arginine and lysine (92). A combination of these two enzymes can therefore be used to detect most of the commonly occurring amino acids if they are C-terminal residues.

C-terminal analysis with CP-A is complicated by the

removal of sub-terminal amino acids as they become exposed; thus a time study of CP-A digestion is frequently necessary to find which residue actually occupies the terminal position. Even such a time study may yield equivocal results as has been clearly demonstrated by the attempts to elucidate the C-terminal sequence of CHTG-A. Four laboratories (28,93,94,116) published different sequences after experiments with CP-A and it was not until Hartley (18) isolated the terminal peptide that the true sequence was found. The results may be obscured even more if the protein contains more than one C-terminus, or if the terminal sequence contains two identical residues. Thus experiments with this enzyme are frequently ambiguous but, in the absence of another, more precise, method, must remain the most general method for examining C-termini.

The use of CP-A in a search for new C-terminal groups produced during activation is somewhat simplified by the fact that no amino acid is produced in molar amounts when CHTG-B is treated with CP-A. Kassell and Laskowski (28) were only able to demonstrate C-terminal asparagine after disruption of the tertiary structure by reduction of the disulfide bonds and preparation of the S-sulfo derivative. Thus in the native zymogen and enzyme the C-terminal asparagine is not available for carboxypeptidase attack.

a. Investigation of conditions for routine C-terminal analysis of CHT-B

In order to carry out routine analysis of samples from

an activation mixture of CHTG-B using a single digestion time with CP-A, conditions must be found under which a maximum of C-terminal groups are liberated. In addition, reagents used to control the course of activation, such as indole, and acylating agents which prevent chymotryptic autolysis during digestion, must have no effect upon the CP-A activity. A high concentration of CP-A was chosen, as Bettelheim and Neurath (42) have found it to be necessary for maximum release of C-terminal leucine from CHT-A.

Two ml of a 0.5% solution of CHTG-B in 0.01 M phosphate buffer, pH 6.7, was activated for 40 minutes at 0° with a zymogen:trypsin ratio of 40:1. A second sample of CHTG-B was activated in exactly the same way, except that the buffer contained 0.01 M indole. Activation was stopped by adding 100 μ l of 1 M TMA-I in acetonitrile and the pH brought to 8.0 with 2 ml of 0.1 M phosphate, pH 8.0. The initial concentration of TMA-I was 2.5×10^{-2} M, which, with an enzyme concentration of 1×10^{-4} M constituted a large excess of acylating agent and, even with the high deacylation and hydrolysis rates recorded in Chapter II, allowed a twofold molar excess after 30 minutes. The temperature of the solution was raised to 25° and 100 μ l of 1% CP-A solution in 10% LiCl, 10^{-3} M DFP, was added to begin digestion.

After 15 minutes and 30 minutes of digestion, 1.0 ml of solution was removed and added to 100 mg of Nalcite HCR resin, 15-30 mesh, in the H^+ form. This treatment lowered the pH to 1.8 and stopped further proteolysis. Shaking the

Table X

Time Study of the Digestion of Chymotrypsin-B
by CP-A at 25° and pH 8

Ratio CHT-B:CP-A = 10:1

Digestion time	mole amino acid per mole protein			
	Indole absent		Indole present	
	Leu	Tyr	Leu	Tyr
15 min.	1.03	0.23	1.00	0.27
30 min.	1.02	0.20	1.01	0.24

tubes for 1 hour ensured adsorption of any free amino acids onto the resin (73). This was washed twice with water and the amino acids eluted with two 2 ml volumes of 6 N NH_4OH . The ammoniacal solutions were dried and any amino acids separated by paper chromatography and quantitatively estimated with ninhydrin as described in Appendix A.

The large amount of C-terminal leucine produced after 15 minutes of digestion does not increase after 30 minutes and remains unaffected by indole (Table X). Therefore, this period of 15 minutes digestion with a CP-A:CHT-B ratio of 1:10 is sufficient to give full release of C-terminal groups. In later experiments, the time has been extended to 60 minutes to ensure complete digestion and A-I has been substituted for TMA-I for reasons given in Chapter II. Clearly, indole is without influence upon CP-A activity, and this result has been confirmed with other synthetic and natural substrates as detailed in Appendix A.

b. Production of C-terminal groups during the activation of CHTG-B

As it has been demonstrated that considerable chymotryptic autolysis follows the initial tryptic activation of CHTG-B, it is of interest to determine what new C-terminal groups are produced during this process. CP-A is a suitable reagent with which to follow these changes as it has a specificity closely parallel to that of CHT-B.

All activations were carried out at 0° with 1% CHTG-B solutions using a ratio of CHTG-B:trypsin of 40:1. The zymogen was dissolved in 0.01 M phosphate buffer, pH 6.7 or

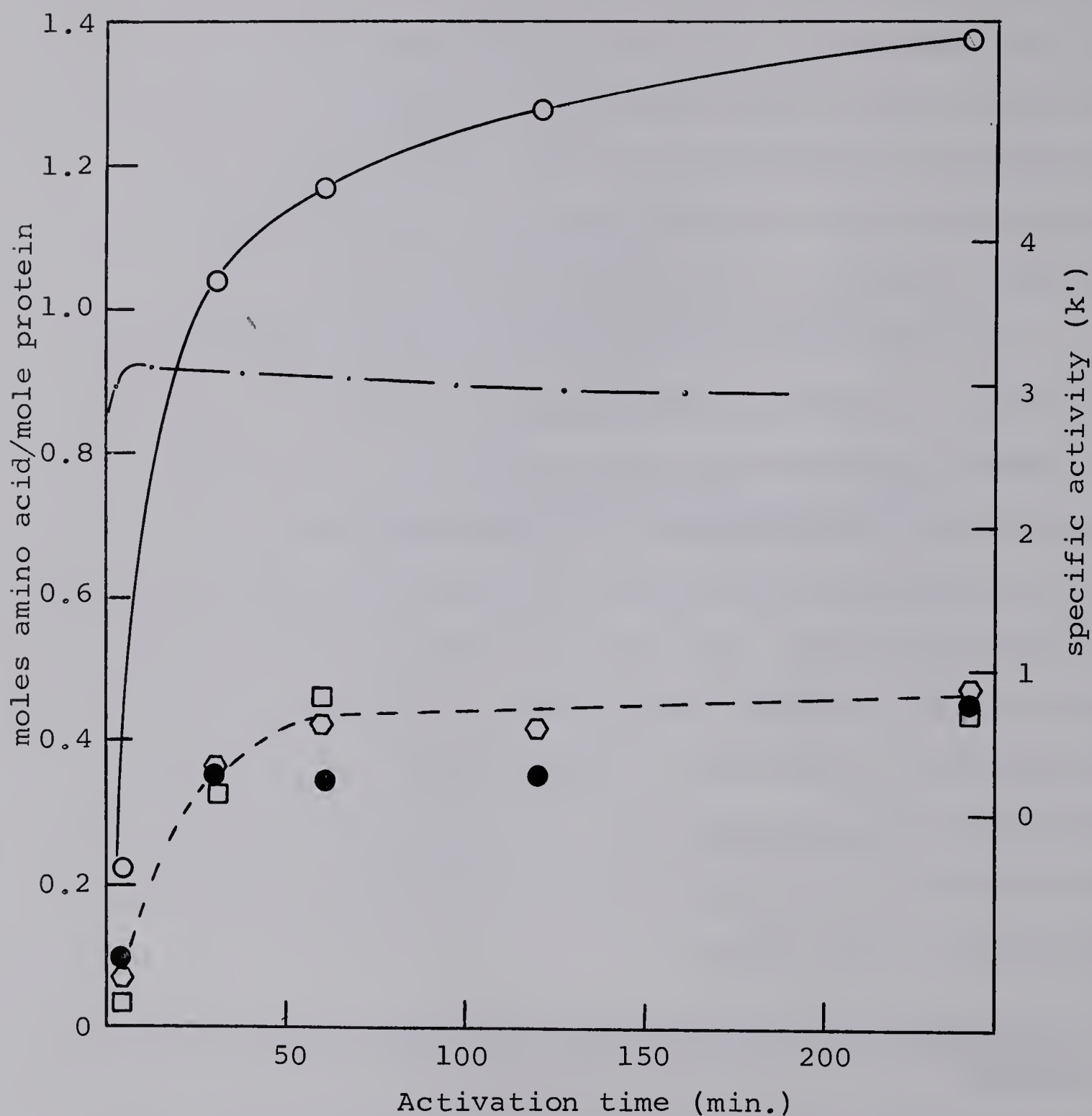


Figure 12. The liberation of new C-terminal groups during activation in 0.1 M Tris, pH 8.0, 0°.

0.1 M Tris-HCl, pH 8.0. The buffer, in some experiments, contained 0.01 M indole or 0.025 M CaCl_2 . Details of each solution are given with the appropriate result. At time intervals during activation, 0.4 μmole samples (1.0 ml of solution) were withdrawn and quick-frozen in dry-ice cooled acetone. Blank samples were taken before the addition of trypsin and frozen in the same way. A small volume (e.g. 100 μl) of zymogen solution was withdrawn and diluted in 5×10^{-3} M HCl for estimation of protein concentration by A_{280} .

Formation of new C-terminal groups was detected by digestion with CP-A. The pH of each frozen sample prepared in 0.01 M phosphate was raised to 8.0 with 0.5 ml of 0.2 M Na_2HPO_4 . Chymotryptic activity was inhibited with 25 μl of 0.1 M A-I in acetonitrile and the samples brought to 25°. Digestion was initiated with 100 μl of 1% CP-A (Worthington Biochemical Corp.) in 10% LiCl, 10^{-3} M DFP. After 1 hour at 25°, 200 mg of Nalcite HCR resin was added to each sample and the free amino acids extracted as described in Part a. Quantitative estimation was carried out on the 50 cm column of a Beckman automatic amino acid analyser.

The considerable amounts of leucine, tyrosine, and phenylalanine produced within the first hour of activation support the earlier evidence for autolysis. The amount of C-terminal leucine increases rapidly during the first 60 minutes of activation as does the yield of both tyrosine and phenylalanine, but thereafter, production is much slower (Figure 12). The amount of autolysis is limited by the inclusion of either indole or Ca^{++} ions in the activation

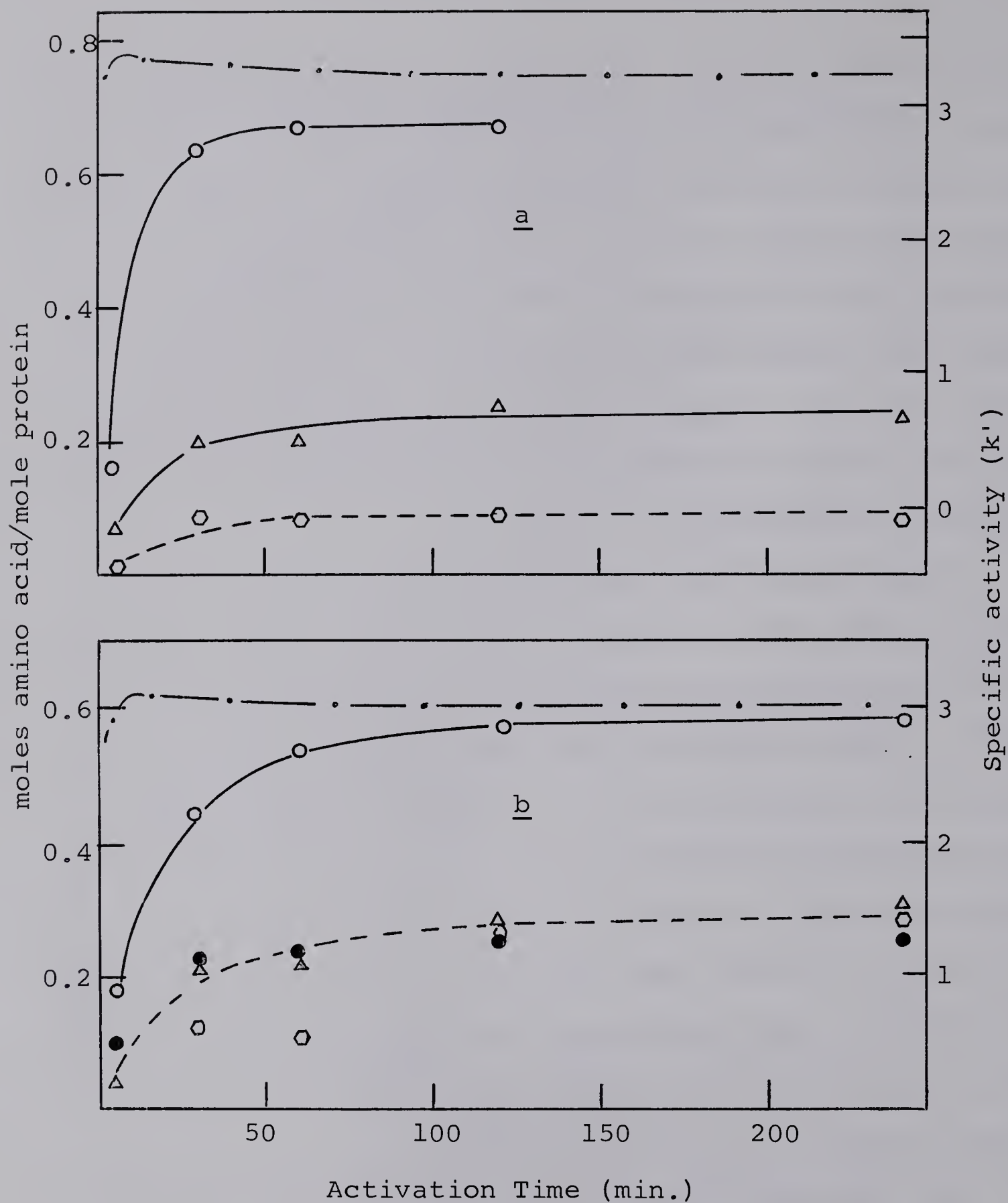


Figure 14. The liberation of new C-terminal groups during activation at pH 8.0, 0° in 0.1 M Tris.

a Containing 0.025M CaCl₂; b Containing 0.01M indole.

-o- Leu; □ Tyr; Δ Phe; -●- Val.

----- Enzyme activity

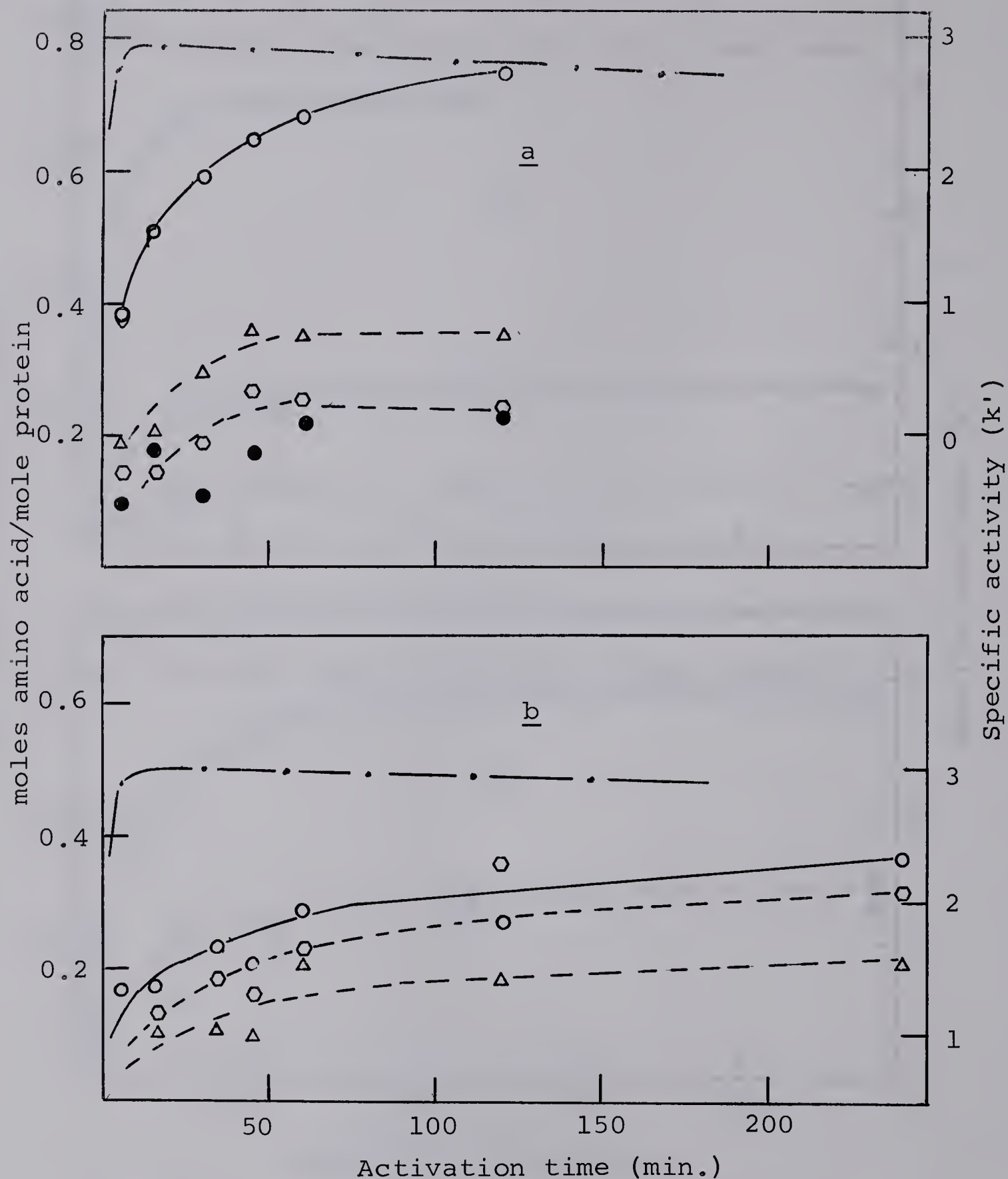


Figure 13. The liberation of new C-terminal groups during activation in 0.01 M phosphate, pH 6.7.

a in phosphate buffer only; b in phosphate buffer containing 0.01 M indole.

○ Leu

◻ Tyr

● Val

△ Phe

Enzyme activity —'—

solution (Figures 13b and 14), confirming that the end groups result from chymotryptic activity. However, the appearance of new C-terminal residues appears to have little relation to the esterase activity, as this value drops very slowly even as considerable amounts of C-terminal leucine are produced. The pattern of C-terminal changes during activation at pH 6.7 and pH 8.0 are essentially the same, except that autolysis proceeds more slowly at the lower pH (Figures 12 and 13a).

c. Time Study of the Digestion of CHT-B by CP-A

Two samples of CHT-B were used. One was prepared by activation of CHTG-B under standard conditions of pH 8.0, 0°, zymogen:trypsin ratio of 40:1, for 2 hours. The pH was lowered to 3.0, the protein dialysed exhaustively against 10⁻³ M HCl and freeze-dried. The second protein sample was Wilson CHT-B which was the same as that used for the N-terminal analyses. It had also been dialysed and freeze-dried.

The enzyme prepared by rapid activation was digested as a 1% solution in 0.01 M phosphate buffer, pH 7.0, 5 x 10⁻³ M A-I, with CP-A. The CHT-B:CP-A ratio was 120:1 and two experiments were carried out, one at 25° and the other at 0°. Digestion of Wilson CHT-B was performed under the same conditions except that the CHT-B:CP-A ratio was 10:1 and a single experiment was done at 25°. Samples were taken during the course of digestion, treated with Nalcite resin, and the yield of C-terminal residues quantitatively estimated with the amino acid analyser.

The results from digestion of CHT-B (2 hour activation)

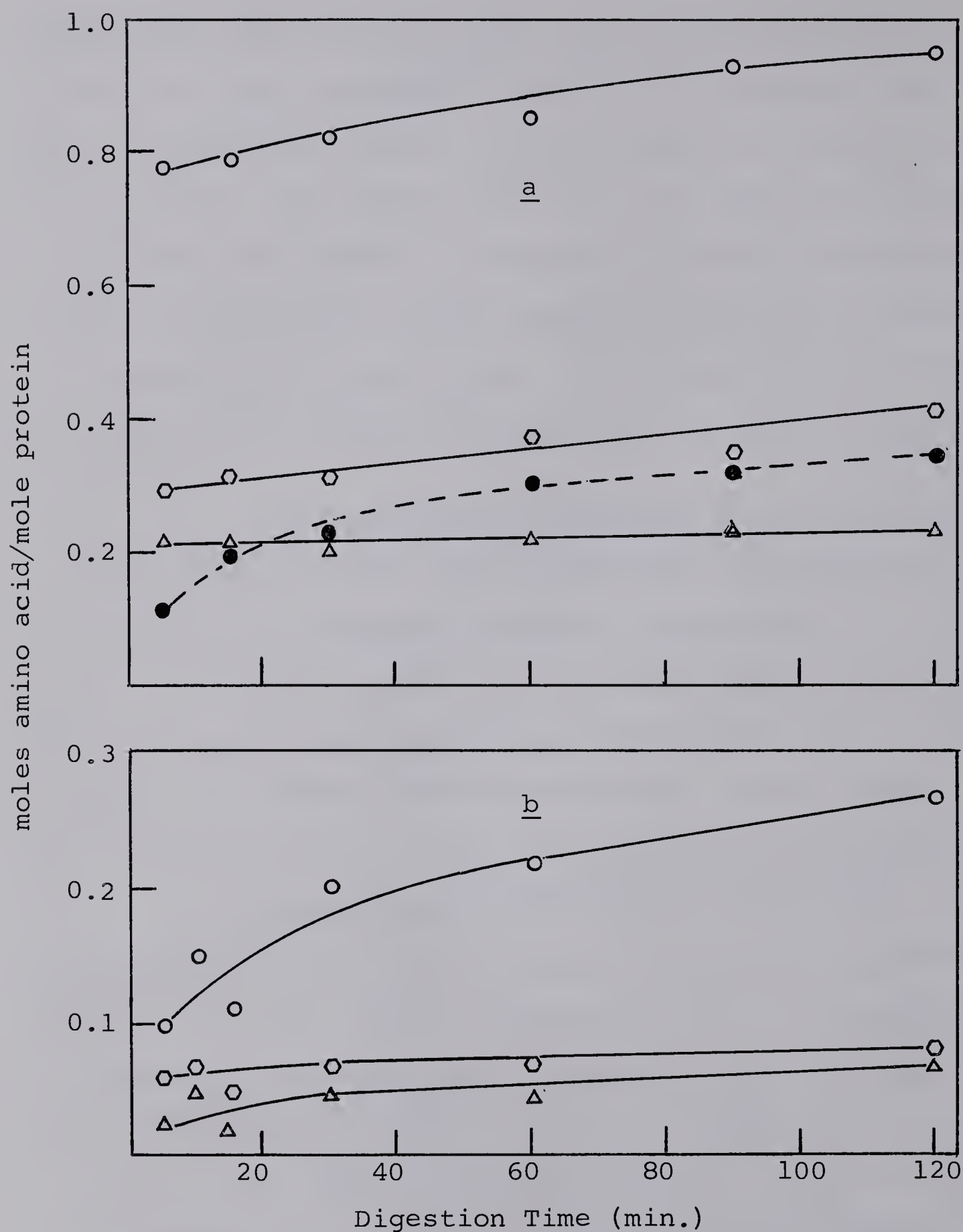


Figure 16. Digestion of CHT-B with CP-A.

CHT-B = 0.5% Ratio CHT-B/CP-A = 120/1
pH = 7.0 (see text for details)

a at 25°

b at 0°

-o- Leu; -○- Tyr; -Δ- Phe; -●- Val.

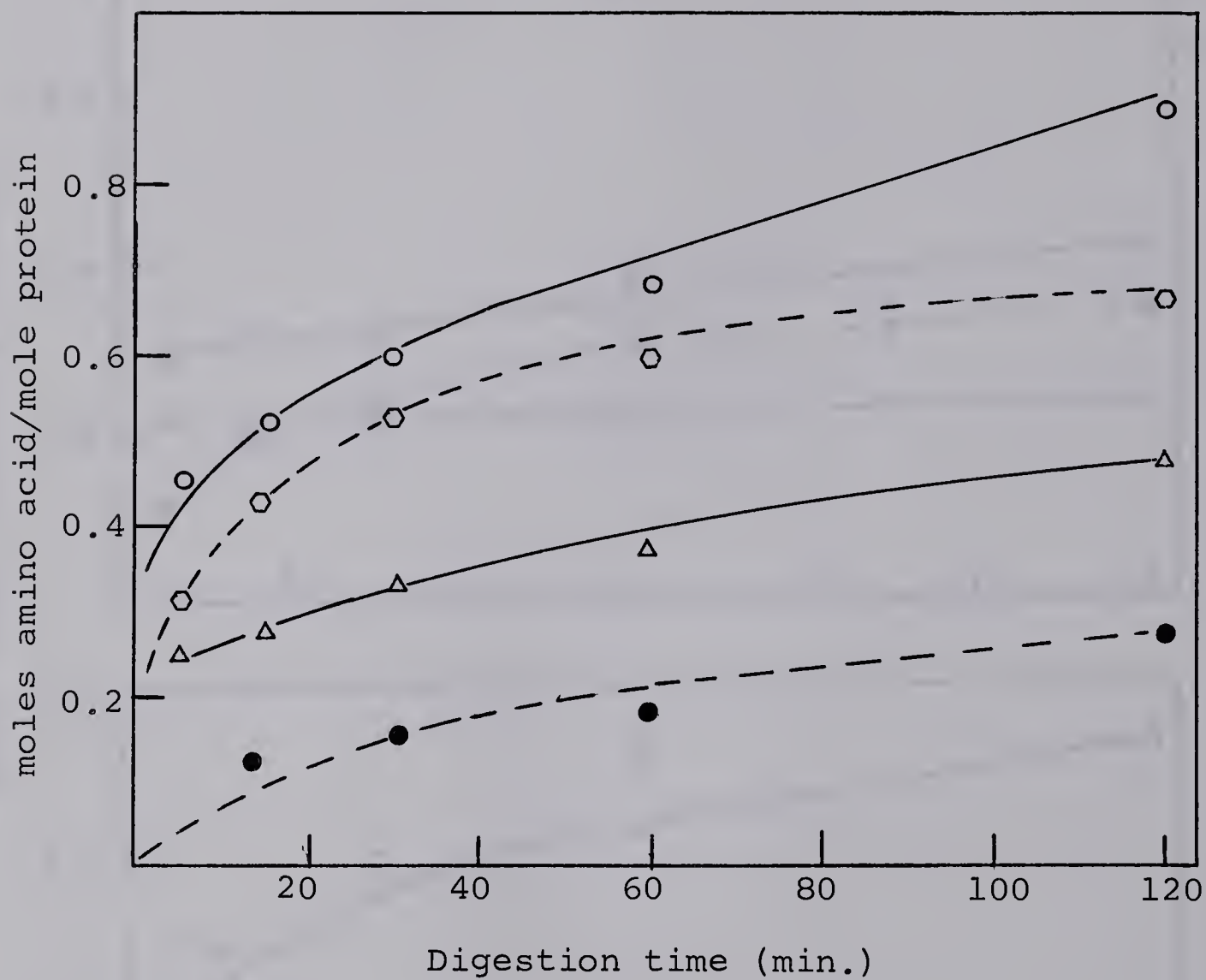


Figure 15. C-terminal analysis of CHT-B (Wilson)
(CHT-B/CP-A = 10/1, 25°)

○ Leu; ○ Tyr; ● Phe; △ Val.

and Wilson CHT-B are quite similar; the major difference lies in the higher tyrosine level in the slow activated enzyme (Figures 15 and 16). All the end groups are produced somewhat more slowly from the Wilson CHT-B than from the rapid activation enzyme. No obvious explanation for this difference is available.

d. Discussion

The production of new C-terminal residues during autolysis may arise in two ways. First, autolysis of a small proportion of the enzyme molecules (perhaps partly denatured) may result in the production of a variety of small peptides in non-molar yields. Since CHT-B has a high specificity towards aromatic and leucine residues (38), the majority of these peptides would be expected to have C-termini of this type. This small fraction of the total protein may thus produce high molar yields of specific terminal groups. Alternatively, the autolysis may involve the bulk of the enzyme molecules present, in which case new C-termini would represent relatively specific cleavages in the polypeptide chain of a considerable proportion of the enzyme molecules present.

The results presented above may represent a situation to which each of these alternative processes is contributing. It has been demonstrated in Part 1 of this Chapter that significant quantities of low molecular weight peptide material are produced by autolysis. Although none of these peptides is produced in molar yields, the net total of new C-termini of any particular type could approach or exceed molar yields.

In this way, any of the C-termini observed, including leucine, tyrosine, phenylalanine and valine, could be derived from this material. This interpretation is perhaps supported by the observation that the yield of C-terminal leucine is reduced by preliminary dialysis of the enzyme preparations (Cf. Figures 12 and 16a).

On the other hand, the appearance of appreciable yields of N-terminal lysine during the autolytic process would indicate that a relatively specific cleavage occurs in the polypeptide chain of the enzyme. For reasons discussed previously, the results of the FDNB method are a reflection of the N-termini of the high molecular weight material rather than of the whole activation mixture. In which case, the appearance of at least one of the C-terminal residues must be due to chymotryptic cleavage of peptide bonds in the polypeptide chain of the enzyme. It is significant that in the primary sequence of CHTG-A and in the partially elucidated sequence of CHTG-B, (see Figure 20) there is the following sequence:

Asn.Asn.Ile.Thr.Leu.Leu.Lys
101 102 103 104 105 106 107

Thus an autolytic cleavage between residues 106 and 107 would yield N-terminal lysine and a larger than molar yield of C-terminal leucine.

It is also possible that some of the apparent C-terminal residues are actually sub-terminal to one of the autolytic cleavage points. This alternative may be examined with reference to the primary sequence of CHTG-B given in Figure 20. It should be emphasized that the placement of the

indicated sequences in this figure is tentative and based only on the positions of corresponding sequences in CHTG-A. However, all three tyrosines (residues 94, 171 and 229), phenylalanines (residues 39, 41, 72, 89, 114, 130 and 144) have been isolated as peptides from tryptic or peptic digests. None of these, with the possible exception of phenylalanine-144, is sub-terminal with respect to another leucine, tyrosine or phenylalanine. Only valine, which is sub-terminal to tyrosine 229, could be accounted for in this manner.

In summary, the N-terminal analyses indicate that two major cleavages occur in the CHTG-B molecule during its activation and subsequent autolysis. The first of these, between arginine-15 and isoleucine-16, is catalysed by trypsin and is associated with the generation of chymotryptic activity. The second, an autolytic cleavage, results in the formation of N-terminal lysine. Digestion with CP-A has not given an unequivocal answer to the identity of the C-terminal residue produced in this step. Significant quantities of C-terminal leucine, tyrosine, phenylalanine and valine have been detected but at least a portion of these could be derived from low molecular weight peptides produced by autolysis of a small fraction of partially denatured enzyme.

4. Isolation and Characterization of the B and C Chains of Chymotrypsin-B

The appearance of new N- and C-terminal amino acid residues associated with the slow decrease in activity during autolysis of chymotrypsin-B suggests the cleavage of peptide bonds in the interior of the polypeptide chain. Since no

small peptides are liberated in molar quantities, at least in the early stages of this autolytic process, the polypeptide chains generated must be held together by disulfide, hydrophobic, or hydrogen bonds. A similar situation exists in the CHT-A₄ molecule, where the A, B and C chains are bound to each other by a total of five inter- and intrachain disulfide bridges. In this case, it is possible to isolate the individual chains, after cleavage of these bonds by reduction and carboxymethylation, by chromatography on DEAE-cellulose in solutions of 8 M urea (18). As it has been shown that the sequences about the disulfide bridges of CHTG-A and CHTG-B are very similar (Smillie and Hartley, J. Mol. Biol., in press) it is reasonable to conclude that the bridges occupy similar locations along the polypeptide chains in both proteins. For these reasons, it appeared likely that the chains presumably produced during the autolysis of CHT-B should be separable by this approach.

a. Reduction and Carboxymethylation

CHT-B was reduced and carboxymethylated by a modification of the method of Canfield and Anfinsen (79). In a typical experiment, 100 mg of protein were dissolved in 10 ml of 0.1 M Tris-acetate buffer, pH 8.0 at 5° and 100 μ l of 1 M DFP in isopropanol added. The solution was left at 5° for 2 hours to convert all the active enzyme to the DIP-derivative. After adjustment to pH 3.0 with 6 N HCl, 6 g of recrystallized urea were added and the temperature raised to 23° to dissolve the urea. The solution was readjusted to pH 3.0 and left at

room temperature for 30 minutes to ensure complete denaturation of the enzyme. Two hundred μ l of 2-mercaptoethanol (Eastman Kodak) was added and the pH raised to 8.0 with 6 N NH_4OH . The tube was flushed with nitrogen, capped and incubated at 37° for 4 hours. After incubation, the solution was transferred, under nitrogen, to a 250 ml polyethylene centrifuge bottle and 100 ml of a mixture of 98% ethanol-2% concentrated HCl (v/v) added. A fine precipitate of reduced protein was produced which was left to develop at -20° for 3 hours before centrifuging at $13,000 \times g$ for 1 hour. The precipitated protein was suspended in 10 ml of 8 M urea, pH 2 and 100 mg of iodoacetic acid (recrystallized from petroleum ether) added. The pH was then raised to 8.6 with 6 N NH_4OH , at which point the protein quickly dissolved, and maintained at this level, under a nitrogen barrier, by the addition of dilute NH_4OH . After 10 minutes, 500 μ l of 2-mercaptoethanol was introduced and the pH maintained at 8.6 for a further 15 minutes to destroy excess iodoacetic acid. The pH was then lowered to 3.0 by the addition of 6 N HCl and the solution dialysed exhaustively against 10^{-3} M HCl. The suspension of precipitated S-carboxymethyl protein (CM-protein) was freeze-dried, and the yield found to be 75% by weight.

Two samples of CHT-B were treated in this manner. One was produced by rapid activation at pH 8.0 for 2 hours and the other was Wilson CHT-B. Each sample was then examined to find the extent of carboxymethylation. As S-carboxymethyl-cysteine is relatively stable to acid hydrolysis, 2 mg of each protein were hydrolysed with 1 ml of 6 N HCl in a sealed

Table XI

Amino Acid Analysis of CM-CHT-B

Results are expressed relative to glycine which is assumed to be present to the extent of 21 residues per mole

Amino Acid	CHTG-B	A'chain ^b	CHTG-B minus A' chain	CM-CHT-B (2 hrs.)	CM-CHT-B (Wilson)
Lys	11	0	11	10.2	10.9
His	2	0	2	1.8	2.1
Arg	5	1	4	3.9	3.9
Asp	20	0	20	19.1	18.3
Thr	20	0	20	21.0	21.3
Ser	21	1	20	17.8	17.4
Glu	19	1	18	17.1	16.8
Pro	13	2	11	11.1	10.9
Gly	23	2	21	21.0*	21.0*
Ala	22	2	20	19.5	19.8
Val	24	2	22	21.7	21.9
Met	4	0	4	3.6	3.7
Ile	8	1	7	7.2	7.2
Leu	19	2	17	14.9	15.5
Tyr	3	0	3	2.8	2.9
Phe	7	0	7	6.7	6.9
CM-Cys	10	1	9	8.2	8.6
Tryp	6 ^a	0	6	(6)	(6)
Total	237	15	222	213.6	215.1

* Arbitrarily fixed.

^aData of Kassell and Laskowski.

^bData of Guy et al.

evacuated tube at 110° for 24 hours. After hydrolysis, the tubes were cooled, opened, and the HCl removed under reduced pressure. The dry residue was taken up in citrate buffer and quantitatively estimated on the amino acid analyser. No dry weights were taken on these samples; instead, the relative amounts of each amino acid were calculated with reference to glycine. Some peptide bonds (e.g. those adjacent to leucine, isoleucine, or valine residues) are resistant to acid hydrolysis, so that the yields of these residues may be below theoretical. Similarly, labile residues, such as serine and threonine, may be expected to be low. Glycine and alanine are generally fully liberated after 24 hours of hydrolysis and are also stable under these conditions. Thus, they may be used as a reference for the calculation of other residues. In the present work, the glycine content of the CM-protein was made equal to the theoretical value of 21 residues per molecule. This figure is arrived at, as shown in Table XI, by subtracting the two residues contained in the A' chain from the total of 23 found in the zymogen. The short A' chain produced by cleavage of the bond between residues 15 and 16 of CHT-B is probably lost during the precipitation of the reduced protein with ethanol-HCl. Its composition and sequence have been independently determined in this laboratory (Figure 20).

Kassell and Laskowski (28), in their work on the amino acid composition of CHTG-B, reported that the molecule contained four cystine residues. The molecule has been re-examined in this laboratory and amino acid analysis after performic acid oxidation have shown it to contain 10 cysteic acid

residues, corresponding to 5 cystines per molecule. If the A' chain is lost, the total number is reduced to 9 half-cystines, and the results of Table XI show that an average of 8.4 CM-cystine residues was actually found corresponding to a 93% yield. This figure is too high to agree with Kassell and Laskowski's value, and therefore, supports the value of 10 half-cystine residues per molecule of CHTG-B observed after oxidation. Clearly, then, reduction and carboxymethylation of the enzyme is virtually complete and attempts to separate the postulated chains may be undertaken.

b. Chromatography

Because of the insolubility of the modified protein, it was necessary to do all chromatography in 8 M urea. Columns of DEAE-cellulose (0.9 meq per g) were prepared in 8 M urea buffered with 0.01 M phosphate, pH 8.0, containing 0.05 M NaCl. All urea solutions were deionized before use by passage through a column of mixed bed ion exchange resin (Dowex 1-50) to remove any cyanate which might react with the protein (77). Chromatography was carried out at room temperature. For analytical experiments, 15 mg of CM-CHT-B was dissolved in 8 M urea and adjusted to pH 8.0. A small sample (50 μ l) was removed and diluted in starting buffer, the A_{280} measured, and the total amount of protein applied to the column calculated. The remainder of the protein solution was charged onto a 0.9 x 70 cm column of DEAE-cellulose, washed in with two 1 ml volumes of starting buffer, and eluted with a linear gradient of NaCl from 0.05 M to 0.14 M.

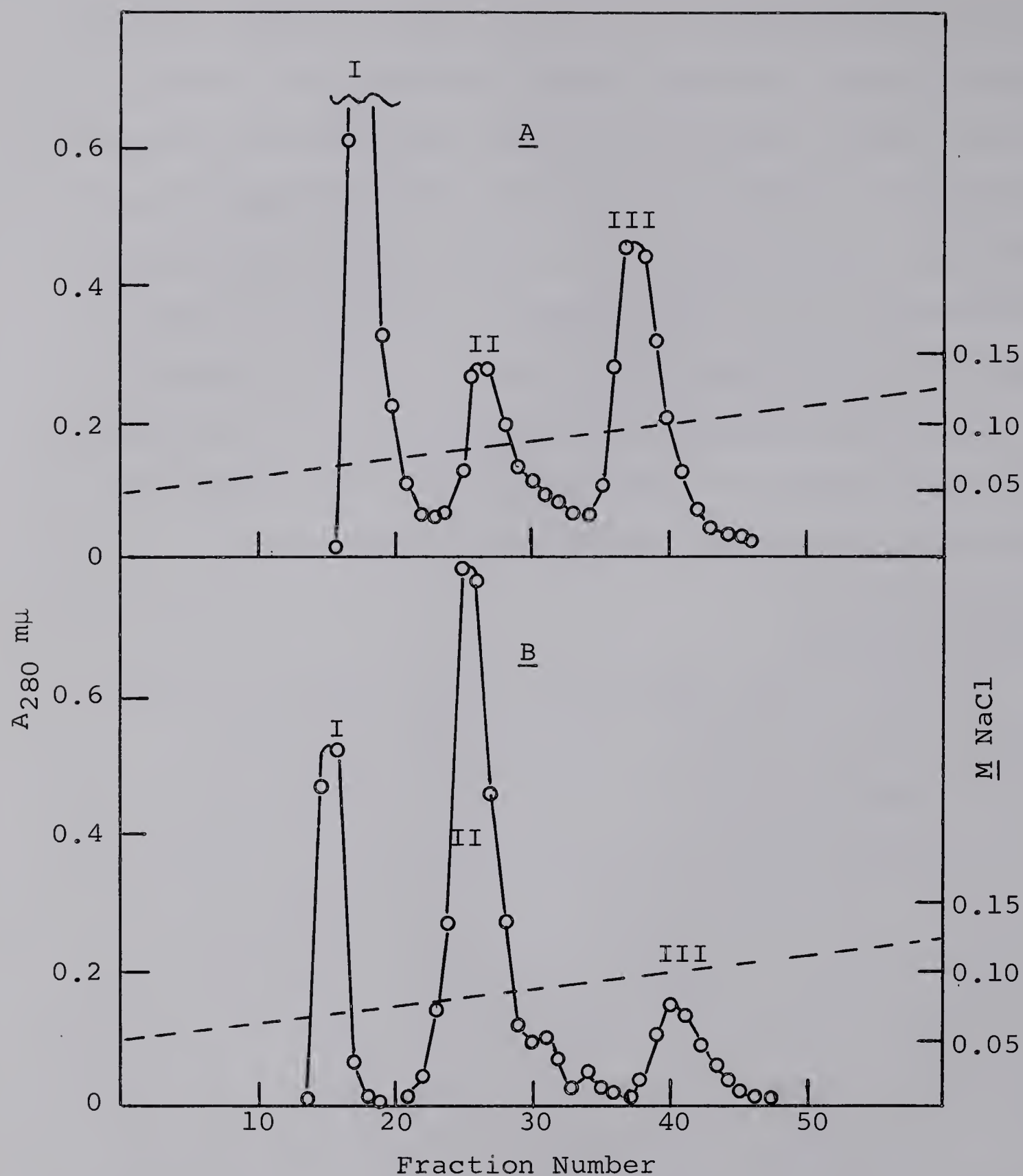


Figure 17. The separation of chains from S-CM-CHT-B (for details, see text).

A CM-CHT-B (Wilson)

B CM-CHT-B (2 hrs. activation)

--- NaCl gradient

- o - A₂₈₀

Two chambers of a Varigrad were used, each containing 100 ml of buffer. The flow rate was 20 ml per hour and 2.6 ml fractions were collected. A recovery of 84% was obtained in the effluent, as measured by A_{280} . Larger diameter columns (2.5 - 3.5 cm) were used for 150-200 mg sample, and the volumes of buffer were scaled up appropriately. The fractions under each chromatographic peak were pooled, dialysed exhaustively against 10^{-3} M HCl and freeze-dried.

The elution patterns from the two types of CHT-B used are shown in Figure 17. In agreement with previous results from terminal analysis and amino acid composition, the two molecules show very similar peak distribution. The major difference lies in the relative abundance of each peak; slow activation produces an enzyme composed mainly of peaks I and III, while rapid activation gives a preponderance of peak II. The percentage of total A_{280} applied to the column which was recovered in peak II from the rapid activation enzyme was 57% while this peak amounted to only 15% of the slow activation protein. It is possible that a correlation exists between the relative amount of peak II and the esterase activity of the parent enzyme, but before such speculation can be entertained, it is necessary to know more about the composition and properties of the individual peaks.

c. Amino Acid Analysis of the Fractions

As material was in short supply, only a single hydrolysis time was used and no dry weights were taken. Amino acid analysis after acid hydrolysis of approximately 2 mg

Table XII

Amino Acid Analysis of Peaks from DEAE Chromatography
of CM-CHT-B
(residues/mole)

Amino Acid	CM-CHT-B (2 hrs. activ.)			CM-CHT-B (Wilson)		
	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III
Lys	4.1	10.4	5.9	3.7	9.6	6.2
His	-	1.9	1.8	-	1.9	2.1
Arg	2.7	3.9	1.1	2.9	3.6	1.1
Asp	7.1	19.6	11.4	7.1	18.0	11.5
Thr	8.7	21.6	11.8	9.4	20.3	11.7
Ser	9.1	18.1	8.5	9.3	16.3	8.6
Glu	4.7	17.7	11.8	5.0	16.2	11.9
Pro	4.8	11.4	6.1	5.2	10.0	6.6
Gly	9.0 ^a	21.0 ^a	12.0 ^a	9.0 ^a	21.0 ^a	12.0 ^a
Ala	9.1	20.4	9.6	9.9	19.4	9.5
Val	8.2	21.8	13.4	8.5	20.5	13.5
Met	2.5	3.8	1.0	2.3	3.2	0.8
Ile	3.0	7.0	4.4	2.8	7.1	5.0
Leu	3.7	16.7	9.4	5.9	15.6	9.3
Tyr	2.1	2.7	0.8	1.9	2.6	0.8
Phe	-	7.1	6.3	-	7.0	6.7
CM-Cys	5.0	7.9	3.5	5.6	8.2	3.4
Total	84	213	119	88	201	121
Apparent mol. wt.	8,500	22,100	12,400	9,000	22,000	12,800

^aArbitrarily fixed.

samples was carried out as previously described (Part a) and the results calculated with reference to glycine as before. Whereas peak II had an amino acid content very similar to the unfractionated protein and is apparently the CHTG-B molecule less the A' chain, peaks I and II showed marked differences, especially in their histidine and phenylalanine contents.

Hartley (18) showed that both of the histidine residues of CHTG-A lie close together at positions 40 and 57, and Walsh and Neurath (113) have described a similar location for the histidine residues of trypsin. Because similarities have been found between the disulfide bonds of CHTG-A and B and both the histidines of CHT-A₄ are located in the B chain, peak III from CHT-B has been tentatively identified as the B chain. Peak I has, consequently, been designated chain C. The total of 21 glycine residues has been divided with 9 allocated to peak I and 12 to peak III. This distribution provides close to theoretical yields, not only of histidine, but of the majority of the other residues.

The approximate length of each chain can be calculated from these amino acid analyses as shown in Table XII. Chain B contains about 120 residues while chain C is 90 residues long. When the 15 residues of the A' chain are added to the B chain, it can be seen that the break in the CHTG-B molecule occurs around residue 135-140, as compared to the position 144-147 in CHTG-A. Allowing for the difference in overall length, CHTG-A contains 246 residues and CHTG-B has 237, the positions appear to be about the same. If the breaks do occur in analogous regions of the two molecules, the

Table XIII
N-terminal Analysis of S-carboxymethyl
Derivatives of CHT-B

Protein	DNP-amino acid (mole/mole protein) ^a		
	Ileu	Lys	Ser
CM-CHT-B (2 hrs.)	0.43	0.28	Trace
Peak I	-	0.10	-
Peak II	0.41	-	-
Peak III	0.09	-	-
CM-CHT-B (Wilson)	0.62	0.49	-
Peak I	-	0.43	-
Peak III	0.43	-	-

^aCalculated on basis of mol. wt. DNP-proteins being:
CM-CHT-B = 25,500; peak I = 11,000; peak II = 25,500;
peak III = 15,000.

distribution of half-cystine residues between the chains should be the same for the B and A proteins. The B chain of CHT-A₄ contains 4 such residues, while the observed number in the B chain of CHT-B is 3.4; the C chains of CHT-A₄ and CHT-B contain 5 and 5.3 residues respectively. Similarly, the B chain of both proteins contain all the phenylalanine residues. It may therefore be concluded that chymotryptic autolysis during slow activation results in breakage of the molecule of both the A and B enzymes in approximately the same region of the polypeptide chain.

d. N-terminal Analysis of CM-fragments

The two chains of CHT-B were further examined in an attempt to determine their terminal residues. N-terminal analysis was carried out after reaction with FDNB. It was observed that the isolated chains reacted slowly with FDNB, so dinitrophenylation in 8 M urea solutions was allowed to proceed for 16 hours at room temperature. A lower concentration of DMF (20%) was necessary in this process to avoid precipitation of the CM-protein. Extraction of the DNP-proteins, hydrolysis and identification of the terminal amino acids was performed as described in Part 2.

Even after 16 hours of dinitrophenylation, no molar yield of DNP-amino acids was found, as can be seen in Table XIII. It is possible that this is due to incomplete dinitrophenylation because the DNP-amino acids which were found are the same as those isolated from the unmodified protein. Hofmann (96) has reported a similar situation in S-amino

ethyl fragments obtained after treatment of trypsinogen with cyanogen bromide. Another explanation could be that the α -amino group is carbamylated and therefore unreactive towards FDNB. It is known that an 8 M urea solution contains 0.02 M cyanate ion (114) and all the reduction, carboxymethylation, and chromatography steps¹ were done in 8 M urea at pH 8.0-8.5. These are the conditions found to be most suitable for carbamylation of amino groups (77). However, if extensive carbamylation has occurred, there should be a loss of lysine due to its conversion to homocitrulline. But, the yields of lysine in Tables XI and XII are close to theoretical. Therefore, the loss of free α -amino groups by carbamylation cannot be high. The finding of N-terminal isoleucine in peaks II and III supports the amino acid analysis which identified them as the B-C fragment (intact polypeptide chain minus A chain) and B fragments, respectively. Peak I gave limited amounts of N-terminal lysine, which confirms its identity as the C chain. Peak II cannot be an aggregate of the B and C chains or N-terminal lysine would have been found.

e. C-terminal Analysis of the CM-peaks

The C-terminal analyses of native CHT-B gave a rather confused picture which was not easily correlated with the N-terminal results. By subjecting the smaller, apparently homogeneous, CM-chains to a similar digestion, it was hoped to arrive at a less ambiguous result.

The CM-proteins were found to have very low solubility at pH 6-9, but were soluble in dilute NH_3 . One μmole of each

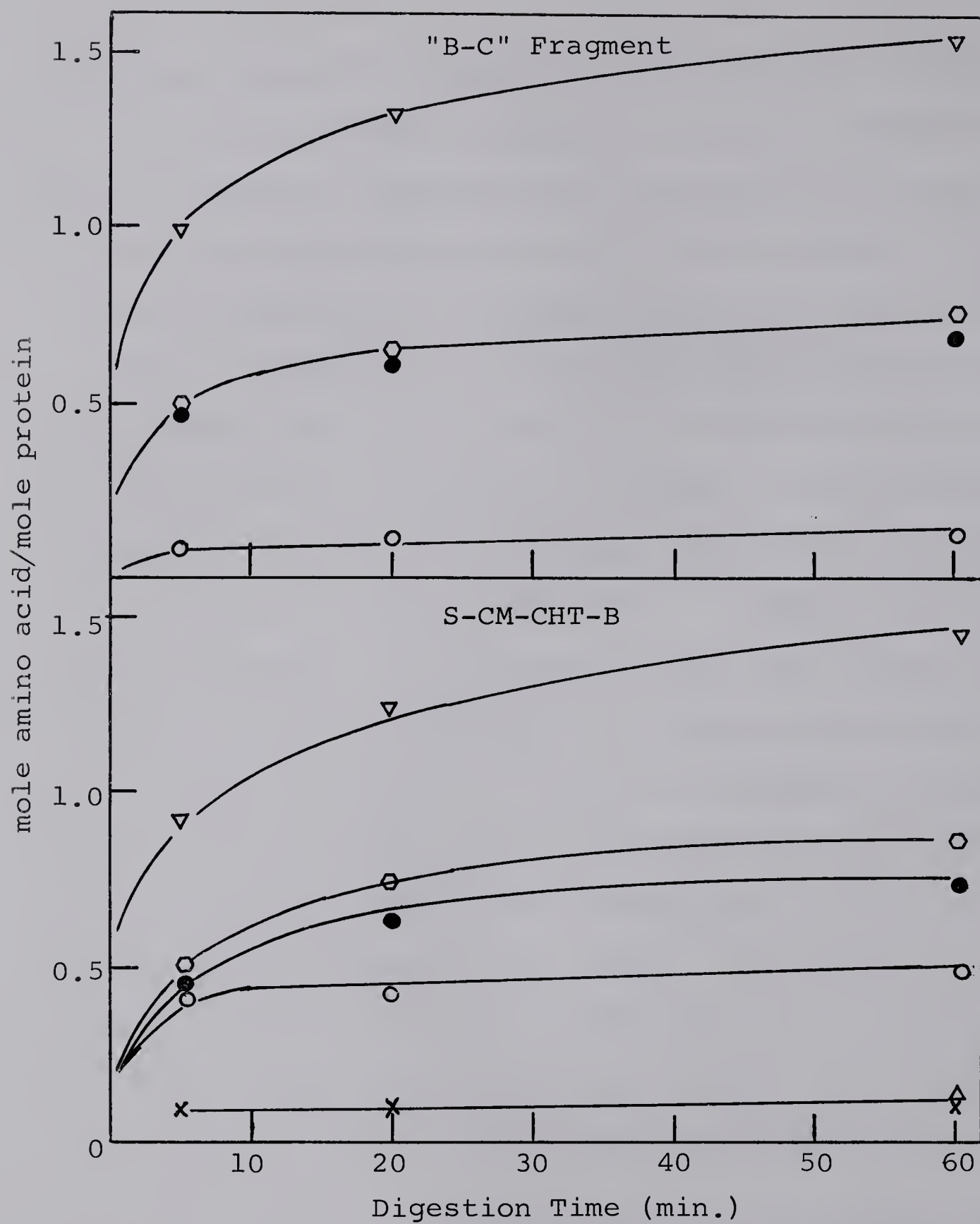


Figure 19. C-terminal analysis of S-carboxymethyl-CHT-B and the S-carboxymethyl "B-C" chain.

● Asp(NH ₂)	▽ Ala
○ Tyr	○ Leu
	x Val

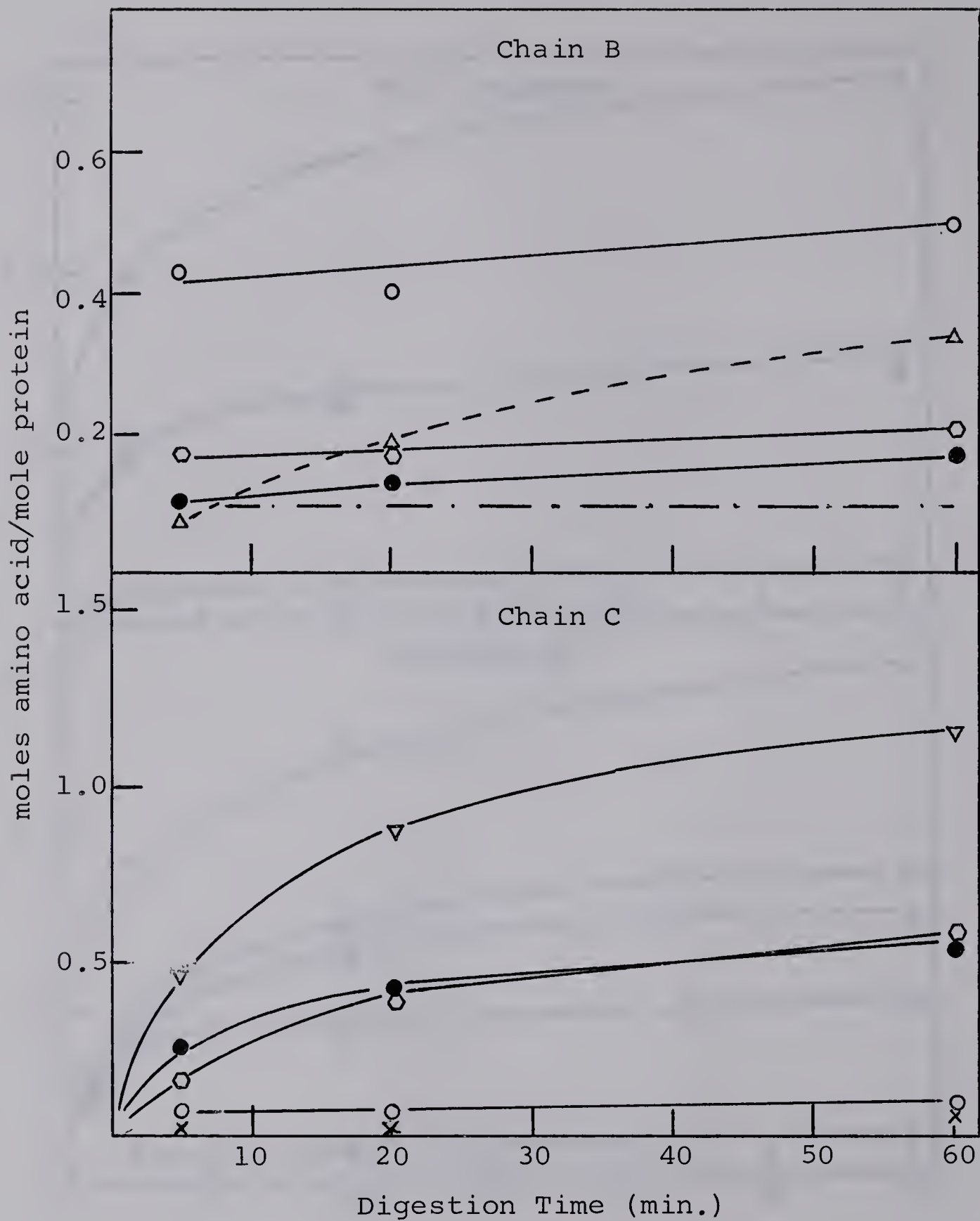


Figure 18. C-terminal analysis of chains B and C from S-carboxymethyl-CHT-B.

○ Tyr	Δ Thr
◊ Leu	▽ Ala
● Asp(NH ₂)	× Val
— · — · — Phe, Ile, Ala.	

derivative was dissolved in 2 ml of 0.01 M NH_4OH and the pH adjusted to 8.5 with 6 N HCl , before adding 100 μl of 0.1 M Tris-HCl , pH 8.0 and 10 μl of 1 M DFP. CP-A (5 mg) was dissolved in 1 ml of 10% LiCl , 0.1 M Tris-HCl , pH 8.0 and treated with 10 μl of 1 M DFP for 30 minutes at 25° . To begin digestion, 25 μl of CP-A solution was added to the protein solution (enzyme:substrate = 1:100). Samples (0.35 μmole) were removed at time intervals and quick-frozen in dry-ice/acetone. Amino acids were adsorbed onto Nalcite resin and quantitatively determined on the automatic amino acid analyser as described in Part 3. In the normal program of the amino acid analyser at 50° , serine and asparagine are eluted in the same position. They may, however, be separated by using a 30° and 50° elution pattern (115). This latter method was used to check the identity of asparagine.

Peak I, which has been provisionally identified as the C-chain, gave mainly asparagine, alanine and leucine, with small quantities of tyrosine and valine (Figure 18). The major amino acid residues appeared in a similar pattern to that found by Kassell and Laskowski for the S-sulfo-zymogen (28) and may be equated with the zymogen C-terminus described for CHTG-A by Hartley (see Figure 20), which has been shown by unpublished work in this laboratory to be identical to the C-terminus of CHTG-B. The small amount of tyrosine and valine found are probably the result of partial cleavage of the tyrosine-alanine bond at position 229-230 (CHTG-A model). Digestion of peak II (Figure 19) gave a very similar result

Table XIV

C-terminal Residues of Various Forms of CHT-B

Protein	C-terminal group, mole per mole protein	
	tyrosine	leucine
Chain B	0.50	0.20
Dialysed Wilson CHT-B	0.59	0.68
Dialysed CHT-B (2 hr. rapid activation)	0.38	0.85
Non-dialysed CHT-B	0.46	1.28

confirming its previous identification as the B-C fragment.

The C-terminal analyses of the B-chain (Peak III) present a more complex picture. After five minutes of CP-A digestion, tyrosine is the major amino acid released (0.43 moles) with smaller amounts of leucine (0.17), asparagine (0.11), threonine (0.08), phenylalanine (0.09), isoleucine (0.09), and alanine (0.09). This relationship is essentially unchanged after 60 minutes of digestion except that threonine has increased significantly to 0.34 moles. From considerations of the present knowledge of the sequence of CHTG-B, as previously discussed, it seems unlikely that tyrosine, leucine or phenylalanine can be liberated by virtue of their sub-terminal position to a chymotryptic cleavage point. Nor can the low molecular weight peptide fraction make a significant contribution to the C-terminal analyses of the B-chain since it was undoubtedly largely removed during the preparative procedures. Thus it must be concluded that the B-chain preparation, although apparently homogeneous with respect to its N-terminus, is heterogeneous with respect to its C-terminus. In this connection, it is of interest to compare the tyrosine and leucine yields from various preparations of CHT-B. C-terminal analyses have been performed on (a) CHTG-B activation mixture, (b) dialysed CHT-B after 2 hour rapid activation, (c) dialysed Wilson CHT-B, and (d) B-chain. The values for 60 minutes digestion with CP-A taken from Figures 13, 16, 17, and 19 are compiled in Table XIV for easy comparison. It is significant that, although the levels of tyrosine remain

roughly constant for each of these preparations, there is a progressive decrease in the amount of C-terminal leucine in going from CHTG-B activation mixture to purified B-chain. The varying levels of leucine in the several preparations can probably be correlated with the expected variable levels of low molecular weight peptide material present. Thus, in a sample taken directly from the activation mixture, such levels would be high. In preparation dialysed prior to C-terminal analysis, the low molecular weight peptides would be reduced and in the purified B-chain such peptides would be essentially eliminated. The C-terminal leucine therefore would appear to arise largely from the C-termini of low molecular weight peptides.

This conclusion may now be considered with reference to what is presently known of the sequence of CHTG-B (Figure 20). Since lysine is the only major N-terminal residue formed during autolysis, a major cleavage must occur at a peptide bond involving the amino group of this amino acid. The greater than molar amounts of leucine found in the C-terminal analyses of undialysed CHT-B indicate that the peptide bond between residues 106 and 107 is cleaved. Thus the C-chain, with an N-terminal lysine and C-terminal asparagine is formed and is recovered in a homogeneous form after reduction and carboxymethylation of the disulfide bridges and chromatography on DEAE-cellulose in 8 M urea. However, at least one additional cleavage must occur on the N-terminal side of the leucine residues 105 and 106. From the relatively

Figure 20. Amino acid sequence of bovine chymotrypsinogen-A (taken from

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Cys	Gly	Val	Pro	Ala	Ile	Gln	Pro	Val	Leu	Ser	Gly	Leu	Ser	Arg	Ile	Val	Gly
Cys	Gly	Val	Pro	Ala	Ile	Gln	Pro	Val	Leu	Ser	Gly	Leu	Ala	Arg	Ile	Val	(Gly

36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
Lys	Thr	Gly	Phe	His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	Asn	Glu	Asn	Try	Val	Val
Ser	Thr	Gly	Phe)	His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	Asn					

71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88
Phe	Asp	Gln	Gly	Ser	Ser	Ser	Glu	Lys	Ile	Gln	Lys	Leu	Lys	Ile	Ala	Lys	Val
												Thr	Lys	Ile	Gly	Lys	Val

106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123
Leu	Lys	Leu	Ser	Thr	Ala	Ala	Ser	Phe	Ser	Gln	Thr	Val	Ser	Ala	Val	Cys	Leu
Leu	Lys	Leu(Pro	Thr	Ala	Ala	Gln	Phe	Ser	Gln	Thr	Val	Ser)	Ala	Val	Cys	Leu	

141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158
Try	Gly	Leu	Thr	Arg	Tyr	Thr	Asn	Ala	Asn	Thr	Pro	Asp	Arg	Leu	Gln	Gln	Ala
(Try	Gly)	Lys	Phe(Ser	Ile	Leu	Thr	Val	Arg)	Thr	Pro	Asp	Lys	Leu	Gln	Gln	Ala	

176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193
Ile	Lys	Asp	Ala	Met	Ile	Cys	Ala	Gly	Ala	Ser	Gly	Val	Ser	Ser	Cys	Met	Gly
Val	Val	Asx	Thr	Met	Ile	Cys	Ala	Gly	Ala(Ser	Gly	Val	Ser)	Ser	Cys	Met	Gly	

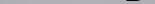
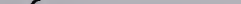
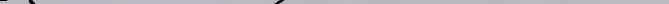
211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228
Gly	Ile	Val	Ser	Ser	Try	Gly	Ser	Ser	Thr	Cys	Ser	Thr	Ser	Thr	Pro	Gly	Val
						Gly	Ser	Ser	Thr	Cys	Ser	Thr(Ser	Thr	Pro	Ala	Val	

Sequences which have been confirmed with "overlap" peptides from trypti only is known are bracketed. Asn indicates asparagine, Gln indicates glu- the dicarboxylic acid residue may be present as the amide or as the free

Hartley [18]) and partial sequence of bovine chymotrypsinogen-B.

19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Asp	Glu	Glu	Ala	Val	Pro	Gly	Ser	Try	Pro	Try	Gln	Val	Ser	Leu	Gln	Asp
Asx	Asx	Glx	Ala	Val	Pro	Gly	Ser	Try	Pro	Try	Gln	(Val	Ser	Leu	Glx	Asx

	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
Thr	Ala	Ala	His	Cys	Gly	Val	Thr	Thr	Ser	Asp	Val	Val	Val	Ala	Gly	Glu	
	Ala	Ala	His	Cys	Gly	Val	Thr	Thr	Ser	Asp							
																	

89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Phe	Lys	Asn	Ser	Lys	Tyr	Asn	Ser	Leu	Thr	Ile	Asn	Asn	Asn	Ile	Thr	Leu
Phe	Lys	Asn	Pro	Lys	Tyr	Asn	Ala	Leu	Lys			Asn	Asn	Ile	Thr	Leu
																

124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
Pro	Ser	Ala	Ser	Asp	Asp	Phe	Ala	Ala	Gly	Thr	Thr	Cys	Val	Thr	Thr	Gly
Pro	Ser	Ala	Asp	(Glx	Asx	Phe	Ala	Ala	Gly	Thr)	Leu	Cys	Ala	Thr	Thr	Gly

159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175
Ser	Leu	Pro	Leu	Leu	Ser	Asn	Thr	Asn	Cys	Lys	Lys	Tyr	Try	Gly	Thr	Lys
Thr	Leu	Pro	Ile	Val	Ser	Asn	Thr	Asp	Cys	Arg	Lys	Tyr	Try	Gly	Ser	Arg

194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
Asp	Ser	Gly	Gly	Pro	Leu	Val	Cys	Lys	Lys	Asn	Gly	Ala	Try	Thr	Leu	Val
Asp(Ser	Gly	Gly	Pro	Leu)	Val	Cys	Gln	Lys								

229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246
Tyr	Ala	Arg	Val	Thr	Ala	Leu	Val	Asn	Try	Val	Gln	Gln	Thr	Leu	Ala	Ala	Asn
Tyr)	Ala	Arg	Val	Thr	Ala	Leu	Met	(Pro	Try)	Val	Gln	Gln	Thr	Leu	Ala	Ala	Asn
→	↔	↔	↔														

and peptic hydrolysis are underlined. Peptides whose composition
tamine, Cys indicates half-cystine. Asx and Glx indicate that
acid.

large amounts of C-terminal tyrosine recovered from all the preparations it is possible that this cleavage occurs at a peptide bond involving the carboxyl group of tyrosine-94. The corresponding amino group would be asparagine. Low yields of DNP-aspartic acid were recovered in the N-terminal analyses (see Table IX). Further, since the C-terminal leucine appears to be largely contributed by low molecular weight material, it would seem that the sequence represented by residues 95 to 106 may be only loosely bound to the rest of the enzyme molecule after cleavage of bonds 94-95 and 106-107, and be lost during the preparation of the B-chain. The low yield of DNP-aspartic acid would be due to the loss of this peptide during the preparation of the DNP-protein as previously discussed.

As Table XIV indicates that a large part of the C-terminal leucine is produced from low molecular weight fragments, it may not be necessary to assume that the C-terminus contains two leucine residues in sequence. The bond between leucine-97 and lysine-98 must also be considered as a possible site for the major chymotryptic split. Only a small tripeptide, Asn-Ala-Leu, separates this site from the tyrosine residue at position 94. Thus cleavage of both bonds would result in the loss of this peptide rather than the 12-residue peptide which separates tyrosine-94 from the Leu-Leu-Lys sequence. The amino acid analyses of the isolated CM-chains show no large losses of asparagine and leucine as would be required if the 12-residue peptide containing three asparagine and three leucine residues were liberated. With the present evidence, therefore,

both sites must be considered as possible points for chymotryptic cleavage.

Such a scheme, however, does not explain all the observations. Additional partial cleavages in the B-chain must occur at a phenylalanine residue and perhaps at a threonine residue. The low yields of apparent C-terminal asparagine, alanine and isoleucine may be due to their sub-terminal positions to one or other of the cleavage points or in part to a low degree of contamination of the B-chain by B-C fragment or C-chain.

No matter which C-terminal leucine is actually exposed during autolysis, the analyses of chain B from a 2-hour activated enzyme indicate that it is heterogeneous with respect to its C-terminus. Some of the molecules have C-terminal tyrosine (50%), others leucine (20%), while the remainder may have residues such as phenylalanine produced by other minor cleavages. It is possible that autolysis may occur at either the leucine or the tyrosine site, but once the first autolytic cleavage has taken place, the second one appears to be hindered. The peptide separating leucine and tyrosine in this region of the molecule must therefore be produced more slowly than the B-chain, as it requires the autolytic cleavage of both bonds. Activation for 2 hours, for example, produces an enzyme which from the A_{280} of the CM-fragment, was found to be composed of 57% B-C chain. Of the 43% of the molecules which had undergone further autolysis, one-half of them showed C-terminal tyrosine in the B-chain, so that only about 0.2 moles of

peptide had been produced for each mole of enzyme. It is unlikely therefore that this peptide could have been present in any quantity in the activation mixtures examined for low molecular weight material.

It is of interest to attempt to correlate the rate of reduction of enzyme activity with the chemical changes described in this chapter. While esterase levels drop slowly during incubation at pH 8.0 and 0° (Figure 6, Chapter III), the autolytic production of new end groups is quite rapid. It may be possible to reconcile these two apparently divergent data if it is assumed that the first form of enzyme (CHT-B₁), which is produced by tryptic cleavage alone, has the same activity as enzyme in which the tryptic and one chymotryptic break has occurred (CHT-B₂). The slower, second major autolytic cleavage can produce a third form of the enzyme (CHT-B₃) and it may be this protein which has a lower enzymic activity.

If a calculation is made using the above scheme, it is necessary to know the activity of each enzyme form. In Chapter III, it was found that the most active enzyme had a k' of 3.3, and this may be taken as the value for CHT-B₁ and CHT-B₂. Slow activation gave an enzyme with a k' of 2.0, which is assumed, for this calculation, to be the activity of CHT-B₃. Thus the drop in enzyme activity in going from CHT-B₂ to CHT-B₃ is 1.3 k' units. After two hours of rapid activation, an enzyme is produced with a k' of 3.0, which is a loss of 0.3 k' units and equal to approximately 20% of the difference between CHT-B₂ and CHT-B₃. C-terminal analyses of the B-chain from enzyme

activated for this time showed, as described above, that only 20% of the total enzyme molecules had undergone both major autolytic cleavages. It therefore appears possible that loss of activity in CHT-B is associated with autolysis at both major sites in the enzyme molecule.

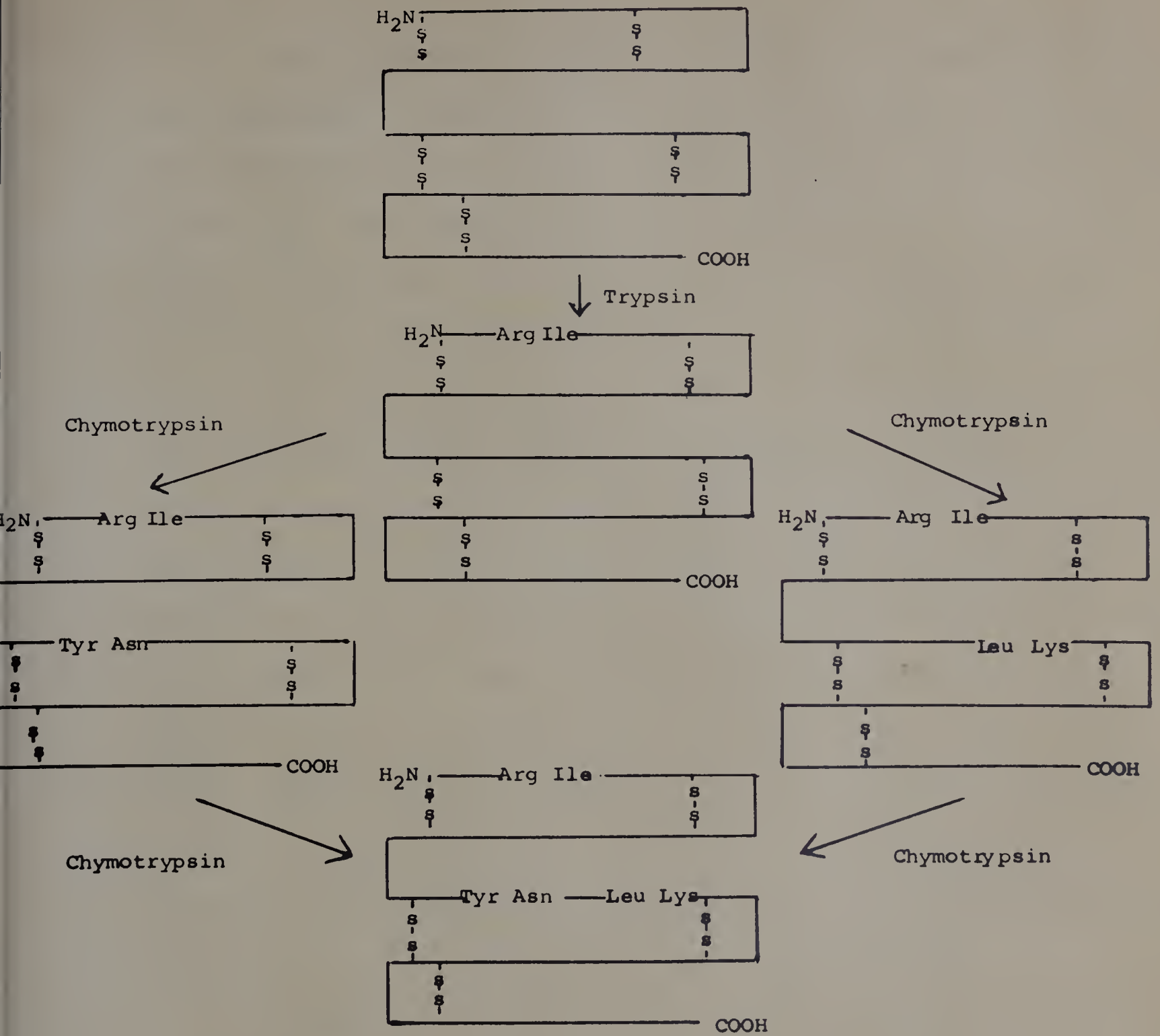
The evidence obtained from the amino acid analysis of the isolated carboxymethyl chains and from the terminal analyses of these chains each give a different location in the molecule for the autolytic cleavages. If it is assumed that the amino acid sequence of CHTG-B is essentially the same as in CHTG-A, then according to Figure 20, the B-chain must contain 80 residues and the C-chain contains 140 residues. However, the amino acid analyses indicate that the B-chain contains 120 residues and the C-chain 90 residues. There is evidence that the tyrosine in position 146 of CHTG-A is absent from CHTG-B and the sequence from residue 143 to 150 is quite different in the two proteins. Also the peptides from tryptic and peptic digests of CHTG-B have been arranged to correspond as closely as possible with those from CHTG-A, but the sequence of the molecule has not yet been definitely established with overlapping peptides. Thus, if the sequence which has been placed between residues 94 and 107 actually is located around residues 140-150 the discrepancy may be resolved. Further work on the structure of the isolated chains of CHT-B should provide a solution to this impasse.

5. Summary

In the first part of this chapter, the results of a search for a specific peptide produced during activation are described. No peptide was found in molar yields although a considerable amount of low molecular weight material could be isolated from zymogen activated for one hour.

The terminal results from the native enzyme are complex and difficult to correlate satisfactorily. It is only after terminal analysis of the isolated S-carboxymethyl fragments that a conclusion may be reached. Following the initial tryptic cleavage of the bond between arginine-15 and isoleucine-16 to form CHT-B₁, at least two major chymotryptic breaks take place. From consideration of what is presently known of the sequence of CHTG-B, it has been tentatively concluded that one of the cleavages is between a leucine and lysine and the second between tyrosine and perhaps asparagine. Since these cleavages are incomplete, the peptide produced is not released in molar yields. Other partial cleavages may occur involving the carboxyl groups of phenylalanine and threonine in the C-terminal region of the B-chain, and between tyrosine and alanine near the C-terminus of the C-chain.

Considering only the major cleavages which have been observed, the results from this chapter may be expressed diagrammatically as follows:



V. PHYSICAL CHEMICAL CHANGES OCCURRING DURING THE ACTIVATION OF CHYMOTRYPSINOGEN-B

The formation of CHT-A from CHTG-A is accompanied by well-documented changes in the physical-chemical properties of the molecule. As early as 1948, Anderson and Alberty (24) noted that the isoelectric point of CHT-A at 8.0 was considerably lower than that of 9.2 observed for CHTG-A. These results were confirmed by Kubacki et al. in 1949 (12) and have since been attributed to the loss of the basic dipeptide Ser-Arg which occurs during the formation of CHT-A₂. Although there is no difference in the molecular weight of the zymogen and enzyme as judged by their $S_{20,w}^0$ values, the zymogen appears to exist largely as a monomer under a variety of conditions of pH and ionic strength (107) while CHT-A₄ has been shown to be in a monomer-dimer equilibrium (25, 83, 84, 103, 104). As the amount of CHT-A₄ in solution is decreased below a critical, pH dependent, concentration, the equilibrium shifts rapidly towards the monomer form, resulting in a non-linear function of $S_{20,w}$ with concentration (80).

Changes in the secondary and/or tertiary structure of CHTG-A upon activation have been studied by the techniques of ultraviolet difference spectrophotometry and optical rotatory dispersion. Chervenka (45) has shown that the generation of chymotryptic activity is closely associated with a decrease in ultraviolet absorption in the region of 287 mμ. From the difference spectra constructed for model compounds (tyrosine

and tryptophane) for which he assumed spectral shifts of 5 Å⁰ towards shorter wavelengths, Chervenka concluded that the observed difference spectrum on activation of CHTG-A was largely attributable to tyrosine. Such difference spectra may arise from changes in the ionic or refractive index environment of the aromatic chromophore (110). They could also, conceivably, arise from the disruption of hydrogen bonds involving the phenolic hydroxyl group. Whatever their immediate cause, these changes are probably associated with an alteration in the conformation of the molecule.

Neurath et al. (49) first reported a change in the optical rotation of CHTG-A and trypsinogen during their activation; an increase in laevorotation at 5890 Å⁰ was observed in both systems. The observations on CHTG-A were later extended by Imahori et al. (48) to cover a range of wavelengths in the visible region and from these results, the authors concluded that there was a doubling of the helical content during activation. More recent data, based on measurements in the ultraviolet region of the Cotton effect, quoted by Schellman (50) are not in agreement with this conclusion. However, the evidence to date strongly indicates that a conformational change of some sort occurs during activation. Whether this involves a change in helical content or is confined to the tertiary folding of the molecule remains an unanswered question at this time.

Information on the changes in the physical properties of CHTG-B during its activation is much more fragmentary and

is restricted to values for the $S_{20,w}^O$ (13,81) and isoelectric points (12) of the zymogen and enzyme. As these data are more than ten years old and were obtained with preparations of protein whose homogeneity is now questionable, it was considered desirable to reinvestigate and extend the observations for comparison with the more recent data on the CHTG-A system.

Initially, it was of interest to examine the sedimentation behaviour of the active enzyme for the purpose of demonstrating, if possible, that the molecular weights of CHTG-B and CHT-A are identical. Both the sedimentation velocity and approach to sedimentation equilibrium methods were used. Secondly, the electrophoretic mobility of the zymogen and enzyme have been examined at pH's on either side of the isoelectric points. Thirdly, possible changes in the conformation of CHTG-B during activation have been investigated by the techniques of ultraviolet difference spectrophotometry and optical rotatory dispersion. These studies show that the conformational modifications associated with the activation of CHTG-B and CHTG-A must be very similar.

1. Sedimentation Studies

a. Methods

(i) Sedimentation Velocity

CHT-B was prepared by activation of a 1% solution of CHTG-B at pH 8.0 with a zymogen:trypsin ratio of 40:1 for 1 hour at 0°. Sedimentation rates were measured on protein dissolved in 0.1 M NaCl, 10^{-3} M HCl, pH 3, and the enzyme

concentration was determined from A_{280} m μ as previously described. Solutions of CHT-B contained in a cell with a Kel-F centrepiece were subjected to a speed of 59,780 r.p.m. in a Spinco model E ultracentrifuge. The temperature was maintained close to 20° with an automatic temperature control device, and photographs (on Kodak metallographic plates) were taken of the schlieren pattern at 16-minute intervals over a period of 64 minutes.

The distance of the concentration gradient peaks from the reference line in the ultracentrifuge cell was measured on a Geartner two-dimensional comparator and corrected for the magnification factor of the optical system ($\times 2.138$). The distance from the axis of rotation was then found by adding 5.72 cm to each value (5.70 cm + 0.02 cm allowed for rotor stretching). The figures were then used to calculate the sedimentation coefficient (s) from the relationship:

$$s = \frac{dx/dt}{\omega^2 x} \quad (7)$$

Equation (7) may be rearranged:

$$s = \frac{2.303}{\omega^2} \frac{(d \log x)}{dt} \quad (8)$$

where dx/dt = rate of migration in cm sec⁻¹, ω^2 = angular velocity in radians sec⁻¹. The sedimentation constant (s) is of the order 10⁻¹³sec⁻¹ and a convenient unit for general use is the Svedberg (S) which is defined as $S = 10^{-13}$ sec⁻¹.

At a speed of 59,780 r.p.m. the term $\frac{2.303}{\omega^2}$ is equal to 9.786×10^{-10} and the second term can be found from the

slope of a graph of $\log x$ versus t . The value obtained under experimental conditions, S_{obs} is usually corrected to 20° in water, $S_{20,w}$ according to the equation (102):

$$S_{20,w} = S_{\text{obs}} \left(\frac{\eta_T}{\eta_{20}} \right) \left(\frac{\eta}{\eta_o} \right) \frac{(1-\bar{v} \rho^o)_{20,w}}{T(1-\bar{v} \rho)_T} \quad (9)$$

The term (η_T/η_{20}) corrects for the viscosity of water at T relative to that at 20° , (η/η_o) is the relative viscosity of solvent to that of water, and the final term is a density correction, where $\bar{v}$ is the partial specific volume of the protein and ρ and ρ^o are the densities of the medium and water, respectively. The value of $\bar{v}$ for CHT-B was taken to be the same as for CHTG-B and equal to 0.733 (23). All the other values were taken from published tables (112).

(ii) Approach to Sedimentation Equilibrium

A 0.8% solution of CHT-B in 0.1 M NaCl, 10^{-3} M HCl, pH 3, was used as in the previous experiments. Two ultracentrifuge runs were carried out, both in a cell with a Kel-F centre-piece. The first was at 59,780 r.p.m. which gave the area of the schlieren peak, and hence a measure of the protein concentration. The second run, using the same stock protein solution was done at a speed of 12,590 r.p.m. Pictures were taken at 16-minute intervals as before, but all the patterns were enlarged tenfold in a photographic enlarger and the new images traced onto paper ruled in mm squares. This manipulation facilitated calculation of the experimental parameters. Calculations were carried out on the meniscus concentration gradient

and at the bottom of the cell where the molecular weight of the protein is defined as:

$$M = \frac{RT}{(1-\bar{v}\rho)\omega^2} \frac{(dc/dx)_m}{x_m c_m} = \frac{RT}{(1-\bar{v}\rho)\omega^2} \frac{(dc/dx)_b}{x_b c_b} \quad (10)$$

M and $\bar{v}$ are the molecular weight and partial specific volume of the protein, c_m is the concentration of protein and $(dc/dx)_m$ is the concentration gradient at the meniscus (x_m cm from the axis of rotation) and c_b and $(dc/dx)_b$ are the corresponding values at the cell bottom. ρ is the solution density, R is the gas constant (8,314 ergs/mole/degree), T is the temperature in $^{\circ}\text{K}$ and ω^2 is the angular velocity.

The concentrations at the meniscus, c_m , and at the cell bottom, c_b , are calculated using the total protein concentration c_o obtained from the sedimentation velocity run according to the expressions:

$$c_m = c_o - \frac{1}{x_m^2} \int_{x_m}^x x^2 (dc/dx) dx \quad (11)$$

and

$$c_b = c_o + \frac{1}{x_b^2} \int_x^{x_b} x^2 (dc/dx) dx$$

where x is a point away from the concentration gradient where $(dc/dx) = 0$ (plateau region). Full details of the calculations are given by Schachman (102).

Table XV

Apparent Molecular Weight of CHT-B* from Approach
to Sedimentation Equilibrium

Centrifugation time (minutes)	Mol. wt. at Meniscus	Mol. wt. at Cell Bottom
48	43,400	37,400
64	42,600	37,600
Average value	40,250 $\pm$ 3,000	

*

As a 0.8% solution in 0.1 M NaCl, 10^{-3} M HCl, pH 3.0.

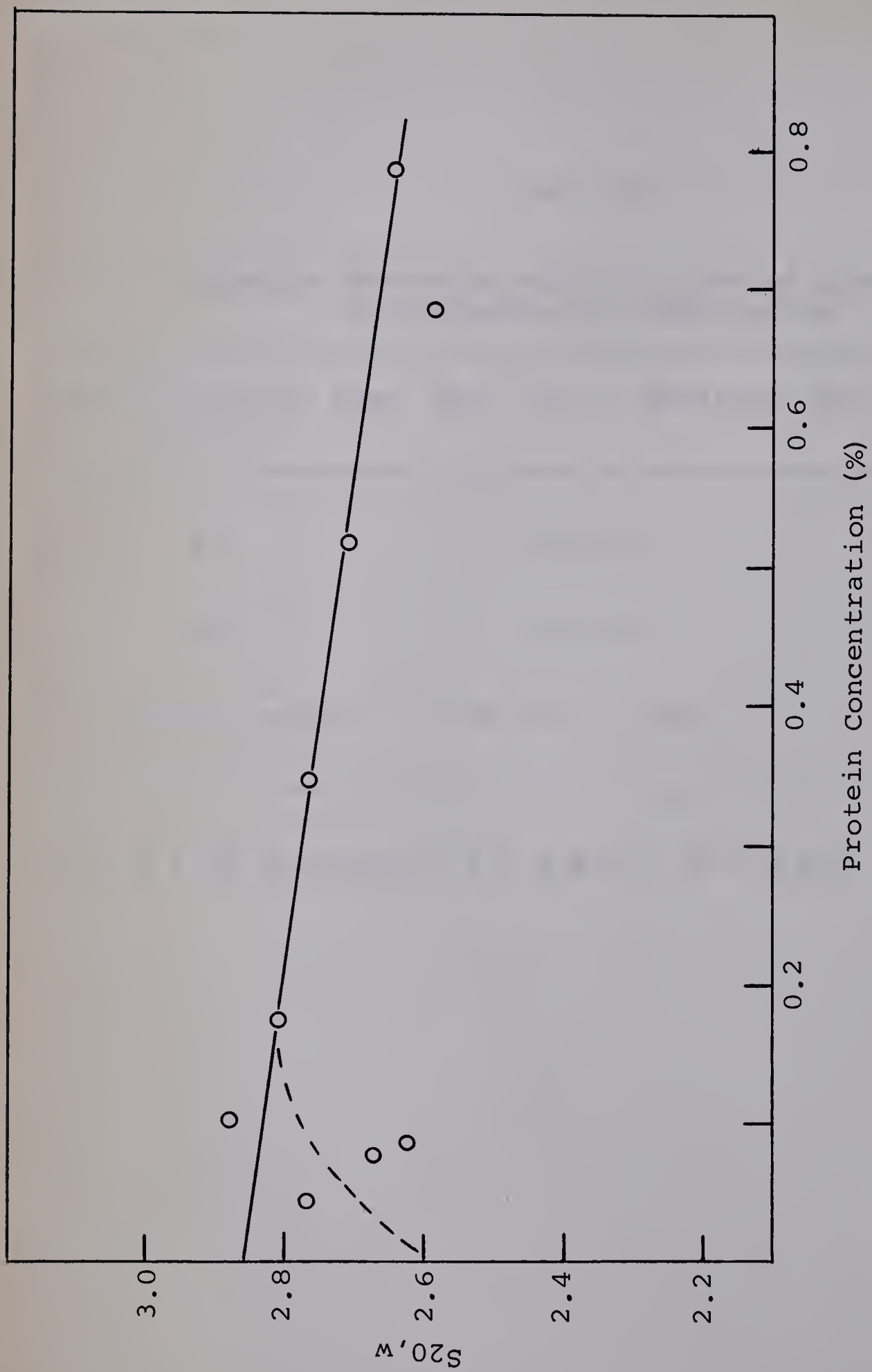


Figure 22

Plot of $S_{20,w}$ vs. protein concentration for CHT-B



a.

b.

c.



d.

e.

f.

Figure 21. Sedimentation velocity run of CHT-B.

a.	0 time,	bar angle	55° ;
b.	16 min.,	"	75° ;
c.	32 min.,	"	50° ;
d.	48 min.,	"	75° ;
e.	64 min.,	"	75° ;
f.	80 min.,	"	75° .

b. Results and Discussion

A typical sedimentation velocity pattern for CHT-B is shown in Figure 21. At all concentrations the protein sedimented as a single major peak. When the sedimentation coefficients were corrected and plotted as a function of concentration over the range 0.04% to 0.08%, the data of Figure 22 were obtained. It can be seen that at concentrations above 0.18% the points fall on a straight line which may be extrapolated to an $S_{20,w}^O$ value of 2.86. The corresponding value of $S_{20,w}^O$ for CHTG-B at this pH is 2.58 (23). However, at concentrations below 0.18% there is a distinct trend, in spite of considerable variability in the experimental points at these low concentrations, towards a lower value of $S_{20,w}^O$. The similar situation which exists for CHT-A₄ has been interpreted by Schwert (25) in terms of a dimerised system at concentrations above 0.4% at pH 3.8 and of a monomer-dimer equilibrium at lower concentrations. The $S_{20,w}^O$ for the dimer form of CHT-A₄ was 36. The value of 2.86 for CHT-B obtained in this study seems to be unreasonable for the purely dimer form of the enzyme. It is conceivable that this figure represents the $S_{20,w}^O$ for a monomer-dimer mixture at concentrations above 0.18%, but it is difficult to understand why the proportions of monomer and dimer are constant as a function of concentration above 0.18% and shift to the monomeric form below this concentration. On the basis of the present experimental evidence there is no ready explanation for these relationships.

While it is unwise, with the limited data available, to make extensive comparisons between the two chymotrypsins, a single determination of the apparent molecular weight of CHT-B by approach to sedimentation equilibrium tends to support the presence of dimer inferred from the sedimentation velocity data. As can be seen in Table XV the average value for the molecular weight of CHT-B at pH 3.0 was found to be 40,250 at a protein concentration of 0.8%. This is considerably higher than the value of 24,500 found for the zymogen under identical conditions.

2. Electrophoretic Studies on Chymotrypsin-B

a. Method

Free boundary electrophoresis was performed in a Beckman, Model H, electrophoresis-diffusion apparatus. Samples of CHT-B (activated for 1 hour under standard conditions at pH 8.0) and CHTG-B were dissolved in acetate buffer of 0.1 ionic strength at pH's ranging from 4.45 to 5.45. Before use each protein solution was dialysed against 2 litres of appropriate buffer for 8 hours. The protein solutions were then placed in a 3.0 ml electrophoresis cell, the electrode vessels filled with buffer, and a current of 6-10 ma passed through the solution at 0°. Migration of the protein was followed by the schlieren optical systems and photographs of the boundary positions taken at time intervals. Conductivity of the recovered protein solution was measured at 0°.

The rate of migration of the descending boundary (dx/dt) was used to calculate the electrophoretic mobility, μ , according to the equation:

$$\mu = \frac{(dx/dt) \cdot K \cdot A}{I} \quad (12)$$

where I is the current, in amps, K is the conductivity in ohms⁻¹ and A is the cross-sectional area of the electrophoresis cell in cm². The mobilities were then plotted against pH and the point of zero mobility taken to be the isoelectric point (IEP).

b. Results and Discussion

In 1949, Kubacki et al. (12) studied the electrophoretic mobility of CHT-B and CHTG-B and showed that the isoelectric

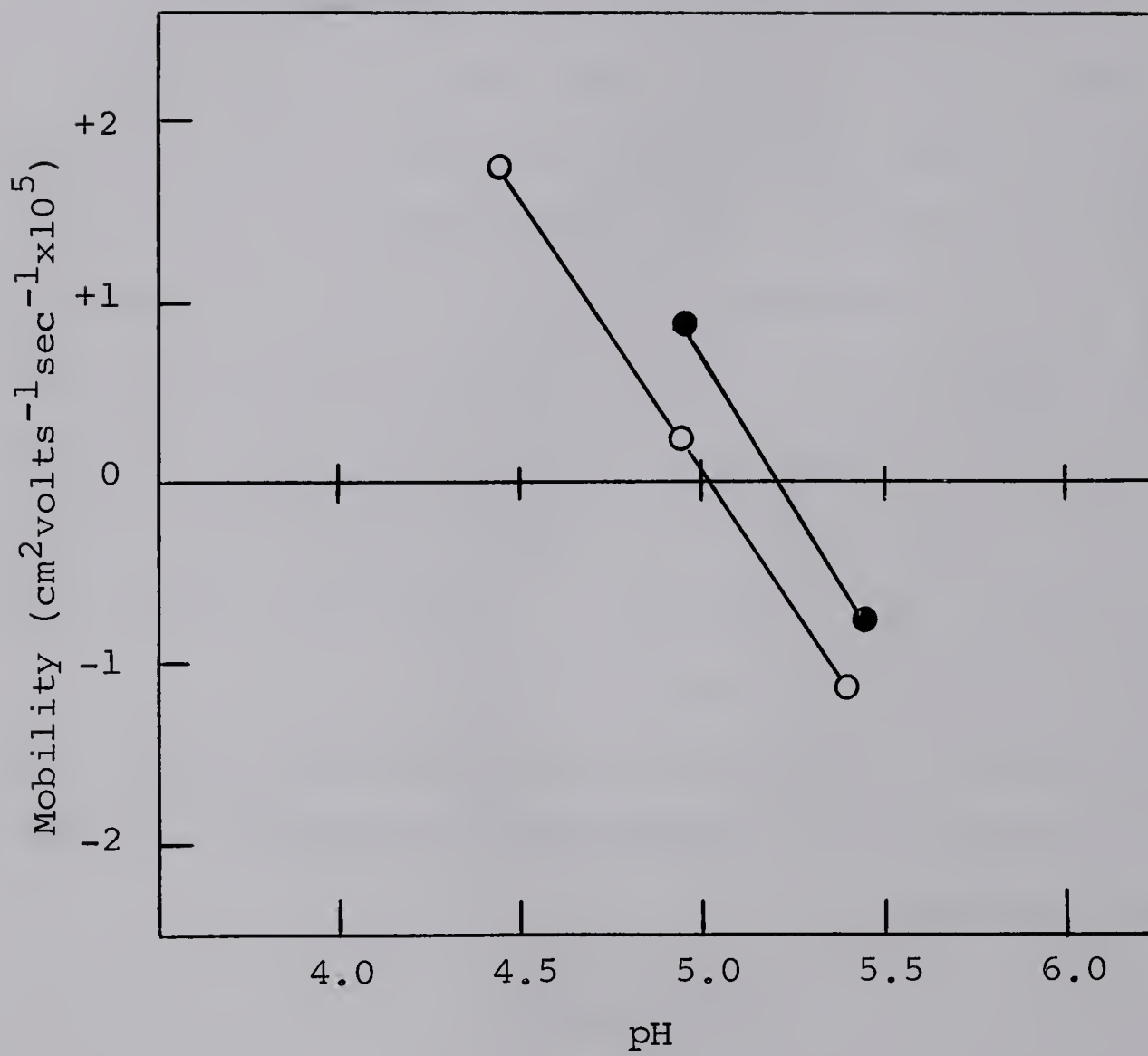


Figure 23. Electrophoretic mobility of CHTG-B and CHT-B.

-○- CHT-B
-●- CHTG-B

points of the two proteins were quite different. As has been shown for the CHTG-A system, the isoelectric point dropped, upon activation, from 5.2 to 4.7. Whereas the change in the CHTG-A system is readily explained by the loss of the basic Ser-Arg dipeptide, no such peptide is liberated during the activation of CHTG-B. The present series of experiments, Figure 23, show that there is only a small change in the isoelectric point, from 5.2 in the zymogen to 5.03 for the activated enzyme and this difference may be accounted for by an alteration in the tertiary structure to expose more carboxyl groups for solvation. Thus the electrophoretic behaviour of CHT-B is in agreement with the known changes in primary structure which accompany activation, while the isoelectric point of CHTG-B is the same as that found by Kubacki et al. (12) and Enenkel (23).

3. Ultraviolet Difference Spectral Studies

The technique of difference spectrophotometry which was first applied to proteins around 1956, greatly facilitates the detection of small spectral perturbations in regions of high absorbance. An increase in analytical precision can be achieved by measuring the absorbance of a concentrated protein solution not against aqueous solvent, but against a reference solution of high absorbance and known concentration. Thus the effect of a particular solvent, or environmental condition may be found by comparing two identical concentrations of the same protein, one in a reference solution and the other in the test solvent. Generally, in this type of work, the absorbance is

kept below 2 to prevent distortion of the spectra and light scattering.

a. Method

All measurements were made with a Beckman DK-2 spectrophotometer equipped with a constant temperature cell housing maintained at 25⁰. The chart range was set at 75-125% transmittance for all experiments. Solutions were placed in four 5 mm light path matched silica cuvettes with two cuvettes in tandem in each light path, according to the following scheme:

Reference path

(a) 1.0 ml of 0.1 M Tris-HCl, pH 8.0

0.1 ml CHTG-B (1%)

(b) 1.1 ml Tris-HCl

10 μ l of 0.2% trypsin

Sample path

(a) 1.0 ml of 0.1 M Tris-HCl, pH 8.0

0.1 ml CHTG-B

10 μ l 0.2% trypsin

(b) 1.0 ml Tris-HCl

The solutions were scanned from 360 m μ to 240 m μ first, for a base line, without trypsin, and then with trypsin in cuvettes (Reference b and Sample a). The scan was begun 5 minutes after adding the trypsin. A second run was carried out with 0.05 M CaCl₂ in the buffer solution.

The point of maximum difference (287 m μ) was used as the wavelength for a time study of activation at 25⁰, following the change in percent transmittance in the presence of

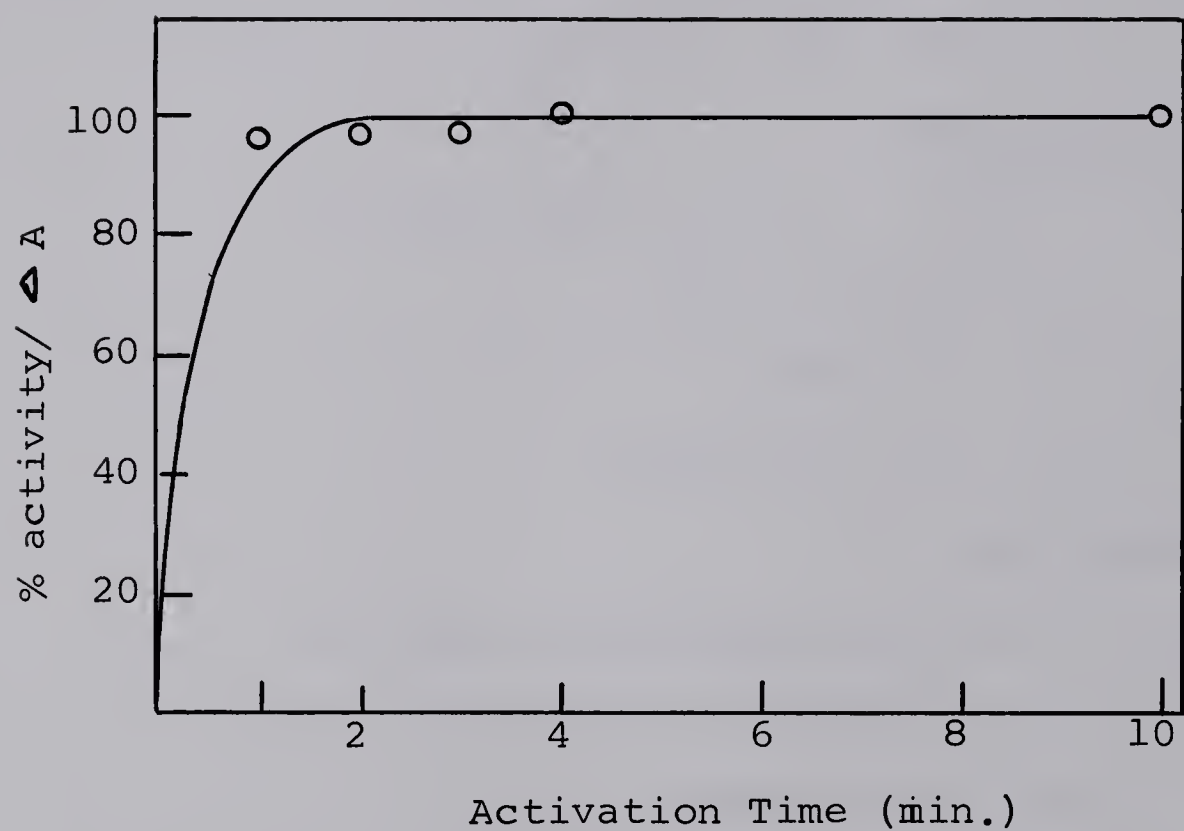


Figure 25. Change in A_{287} during activation of CHTG-B.

— ΔA_{287}
 ○ Esterase activity

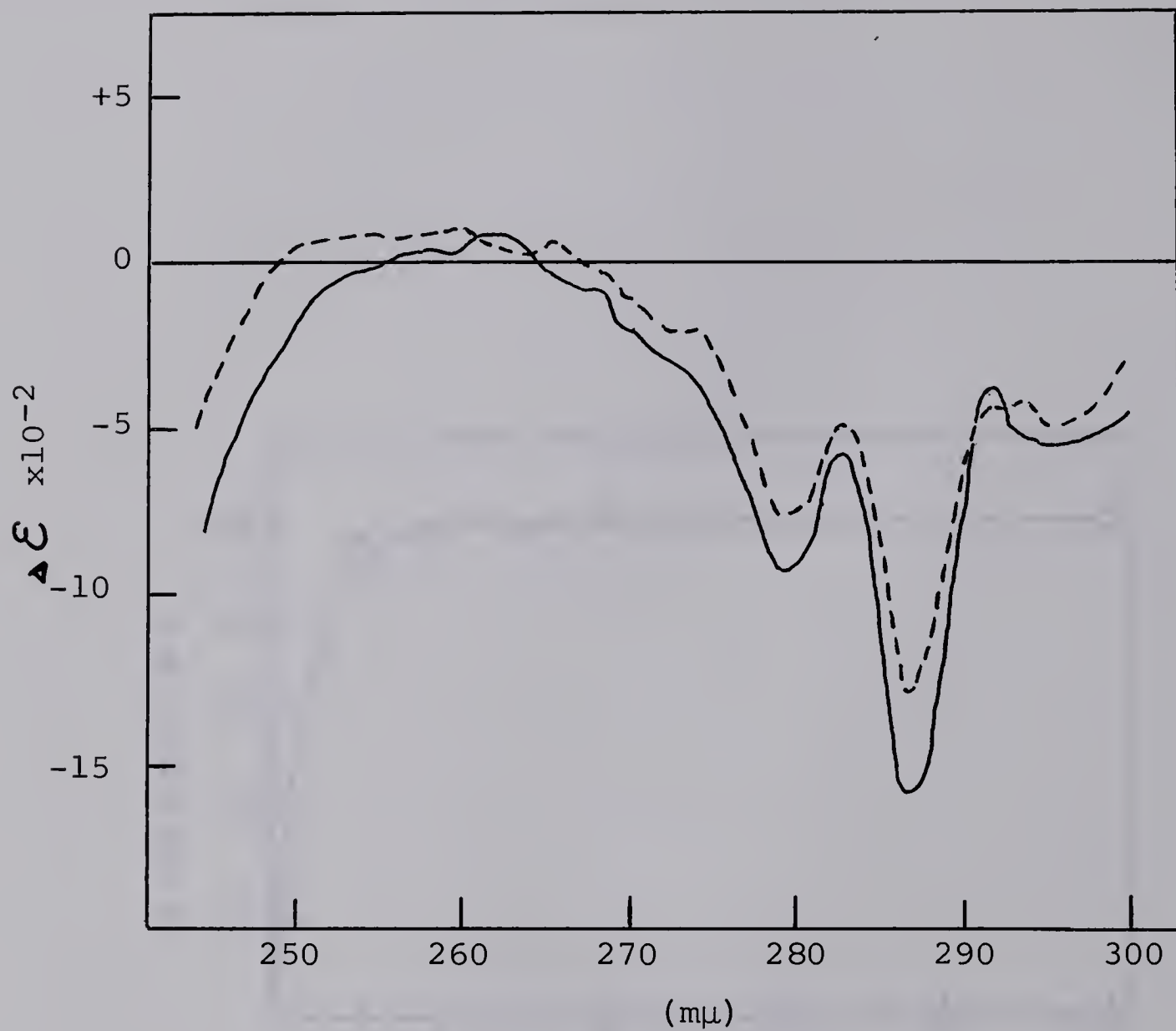


Figure 24. Difference spectra of CHT-B versus CHTG-B.

----- No Ca^{++}
 ——— in 0.05M CaCl_2

0.05 M CaCl_2 . The four cuvettes were arranged in the same manner as for the previous experiment. Parallel determinations of esterase activity were carried out in the pH-stat as previously described.

b. Results and Discussion

Conformational changes in a protein may alter the micro environment of one or more of the aromatic residues and cause a shift in the absorption spectrum. Chervenka (45) found a spectral shift after CHTG-A was activated by trypsin and Hashizume and Imahori (39) demonstrated a similar change when CHTG-A was activated by pronase. Although Smillie (111) was unable to detect any spectral shift during the activation of trypsinogen, a difference spectrum of the same type as that found by Chervenka after CHT-A autolysis was apparent after tryptic autolysis had taken place.

The spectral changes accompanying activation of CHTG-B (Figure 24) are similar to those observed for CHTG-A, in that there is a sharp trough at 287 $\text{m}\mu$. The magnitude of this trough was found to be $900 \text{ l mole}^{-1}\text{cm}^{-1}$ by Chervenka, and $10^3 \text{ l mole}^{-1}\text{cm}^{-1}$ by Hashizume and Imahori for the CHTG-A system while the difference was $1.3\text{-}1.6 \times 10^3 \text{ l mole}^{-1}\text{cm}^{-1}$ for the CHTG-B system. The close association of changes in A_{287} with activation of CHTG-B is illustrated by Figure 25. It was shown in Chapter III that CHTG-B is activated more rapidly than CHTG-A and these results confirm the findings. A similar experiment carried out by Chervenka on CHTG-A indicated that full activation required 120 minutes, while

CHTG-B was activated in 2 minutes. The position of the minimum of the difference spectrum at 287 m μ implicates tyrosine as the major contributant to the spectral perturbation. The shift may be ascribed to a modification of the micro environment of at least one such residue, probably resulting from a change in the tertiary structure of the molecule. While no quantitative correlation of the difference spectrum with tertiary structural changes is yet possible, these results do show that both CHTG-A and CHTG-B undergo similar changes during activation.

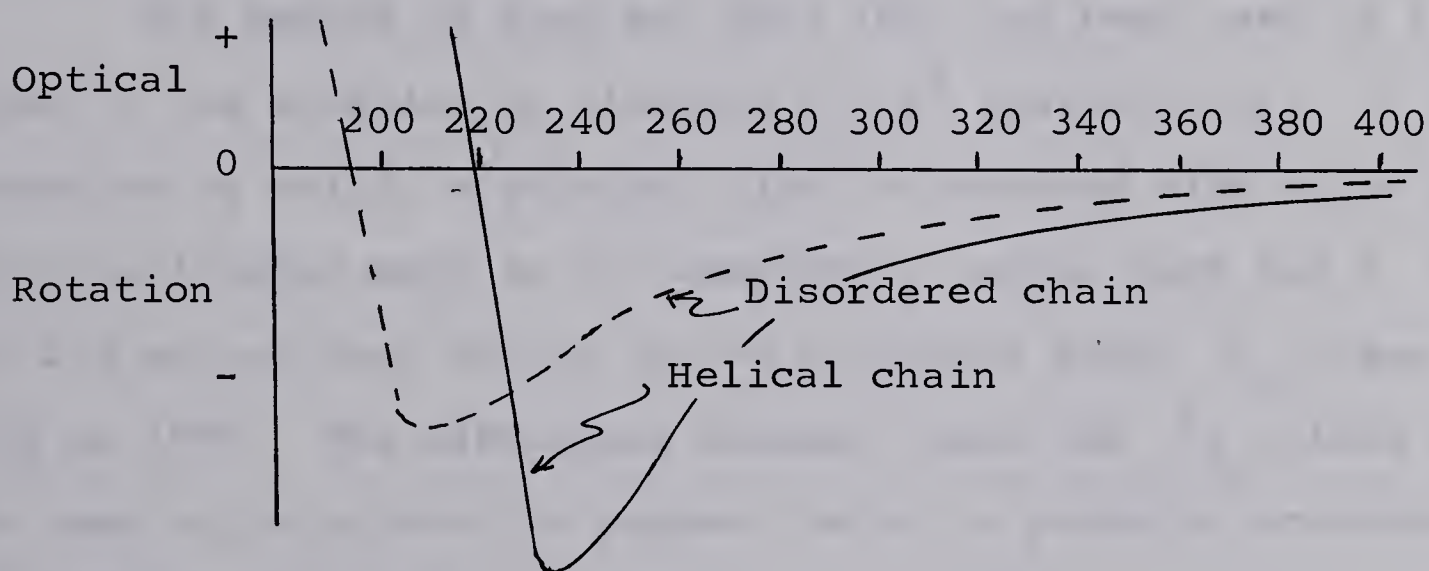
4. Optical Rotatory Dispersion

Rotation of the plane of polarized light by an asymmetric centre in a molecule is a well-known phenomenon. In proteins, optical rotation is the result of two principal factors, the asymmetric α -carbon of the constituent amino acids and the asymmetric arrangement of the polypeptide backbone (the conformation). Optical rotation phenomena originate, as do spectral absorption characteristics, from the excitation by light of charges within a molecule, but optical rotation may be dispersed over a much wider wavelength range than the corresponding absorption band. It is thought that the optical rotatory dispersion displayed by proteins throughout the visible region is associated primarily with absorption bands in the ultraviolet region (at 145 m μ , 185 m μ , and 225 m μ).

The rotation is quite small in the visible region but as measurements are extended to shorter wavelengths, it becomes larger, passing through a trough at 233 m μ , then

rising sharply through zero to a large +ve peak at 190 mμ. The positions of the Cotton effect peaks are associated with absorption bands of the peptide chain and the magnitude of the trough at 233 mμ, and the visible rotation can be correlated with the conformation of the polypeptide chain. In a disordered chain, the visible rotation becomes less negative and the trough at 233 mμ is lost, giving rise, in synthetic polyamino acid systems, to a new smaller trough at 204 mμ (101).

These changes in conformation may be followed by measuring optical rotation in the visible and ultraviolet regions of the spectra. The expected changes can be summarized diagrammatically:



Although optical rotation has been used for many years as a tool for measuring protein conformation, it is only since 1950 that advances in instrumentation have permitted rotation measurements to be made over a range of wavelengths within the visible region. These were not extended into the ultraviolet region below 3000 Å until 1960. For purposes of analysis, it is convenient to treat the data from the two regions of the spectrum separately.

Optical Rotatory Dispersion in the Region 300 mμ to 600 mμ

The Drude equation

The one-term expression known as the Drude equation (97) has been found applicable to proteins and polypeptides having a low helical content, and takes the form:

$$[\alpha]_{\lambda} = \frac{A}{\lambda^2 - \lambda_c^2} \quad (13)$$

where $[\alpha]_{\lambda}$ is the specific rotation at wavelength λ , A is a constant, including correction for refractive index, and λ_c is the mean wavelength of the absorption bands contributing to the visible rotation.

The method of Yang and Doty (98) has been used to fit data to the equation by plotting $[\alpha]_{\lambda} \lambda^2$ against $[\alpha]_{\lambda}$. If the equation is valid, a straight line is produced with slope λ_c^2 . Poly-L-glutamic acid in the completely random form has a λ_c of 212 mμ and when 40% is in the α-helical form, λ_c rises to 268 mμ (99). The difference between these two λ_c values can be used to calculate the percent helix in proteins according to the relationship:

$$\frac{\lambda_c - 212}{268 - 212} \times 40 = \% \text{ helix} \quad (14)$$

The Moffitt equation

Since the Drude equation is not suitable for general application to all polypeptides, especially those with high helical content which show a complex optical rotatory dispersion, Moffitt (100) developed a more general equation:

$$[m']_{\lambda} = \frac{a_o \lambda_o^2}{\lambda^2 - \lambda_o^2} + \frac{b_o \lambda^4}{(\lambda^2 - \lambda_o^2)^2} \quad (15)$$

where $[m']_{\lambda}$ is the reduced mean residue rotation. This value incorporates terms for refractive index correction ($\frac{3}{n^2+2}$, where n is the refractive index of the solvent) and the mean residue rotation, $[m]$, which is $\frac{\text{mean residue weight}}{100} [\alpha]_{\lambda}$. The mean residue weight for most proteins is 115, but that for CHT-B is 106. The constant, λ_o , is generally taken as 212 $m\mu$ for most proteins and polypeptides. However, for certain systems, it is necessary to choose a higher or lower value of λ_o to achieve linearity in the plots. The value of 212 $m\mu$ was found to be satisfactory for the present work. The constants a_o and b_o relate directly to the backbone configuration of the polypeptide chain. a_o is dependent on the interaction of residues in the three-dimensional structure and is also dependent upon residue-solvent interaction. Changes in the a_o of a protein in a constant environment may therefore be taken as an indication of changes in its tertiary structure. The b_o of a protein may be taken as a measure of its helical content. Such estimations are based on empirical studies with polyamino acids which have shown b_o to vary from close to zero for random coil structure to -640° for structures containing 100% right-handed helix. On the assumption that no other ordered structures, i.e. left-handed helices or β structures, are contributing to the value of b_o and that the relationship between b_o and percent helix is a linear one, b_o can be used to calculate the helical content. For a fuller discussion of

these relationships, the reviews by Schellman and Schellman (50), Urnes and Doty (99) and Fasman (97) may be consulted.

A graphical solution for the Moffitt equation, according to Urnes and Doty (99), is obtained by plotting $-[m']_{\lambda} \frac{\lambda^2 - \lambda_o^2}{\lambda_o^2}$ against $\frac{\lambda^2}{\lambda^2 - \lambda_o^2}$. The slope of the line is b_o , while the intercept with the $-[m']_{\lambda} \times \frac{\lambda^2 - \lambda_o^2}{\lambda_o^2}$ axis is a_o . Percent helix is calculated by dividing the b_o value obtained by -640° .

Optical Rotatory Dispersion in the Ultraviolet Region

Percent helix from measurements in the ultraviolet region of the spectrum is calculated from the value of $[m']$ at 233 mμ, which is $-12,700^\circ$ for 100% helix and -1800° for a random chain (97). Thus the observed rotation may be used to calculate $[m']_{233}$ and, from this, the percent helix can be derived according to the relationship (104):

$$\frac{([m']_{233} - 1,800^\circ) \times 100}{12,700^\circ} = \text{percent helix} \quad (16)$$

Values of n , the solvent refractive index, may be obtained from tables such as those published by Fasman (97).

a. Method

(i) Visible Region

Solutions of protein (0.3% in 0.1 M KCl, 10^{-3} M HCl, pH 3.2) were placed in a 0.5 dm polarimeter cell with quartz end windows and the cell transferred to a manual spectropolarimeter (O. C. Rudolph & Sons, Inc., Model MSP-4) for measurements of the optical rotation over the wavelength range 310 mμ

Table XVI

A Summary of the Data Obtained from Optical Rotatory
Dispersion Measurements on CHTG-B and CHT-B

Method of Analysis	CHTG-B	CHT-B
Yang-Doty	$\lambda_c = 232 \text{ m}\mu$ % helix = 14.2	$\lambda_c = 232 \text{ m}\mu$ % helix = 14.2
Moffitt	$a_o = -424^\circ$ $b_o = -104^\circ$ % helix = 16.3	$a_o = -340^\circ$ $b_o = -97^\circ$ % helix = 15.1
Cotton effect	$[m']_{233} = 3,466^\circ$ % helix = 13.1	$[m']_{233} = 3,317^\circ$ % helix = 12.0
Average percent helix	14.5	12.6

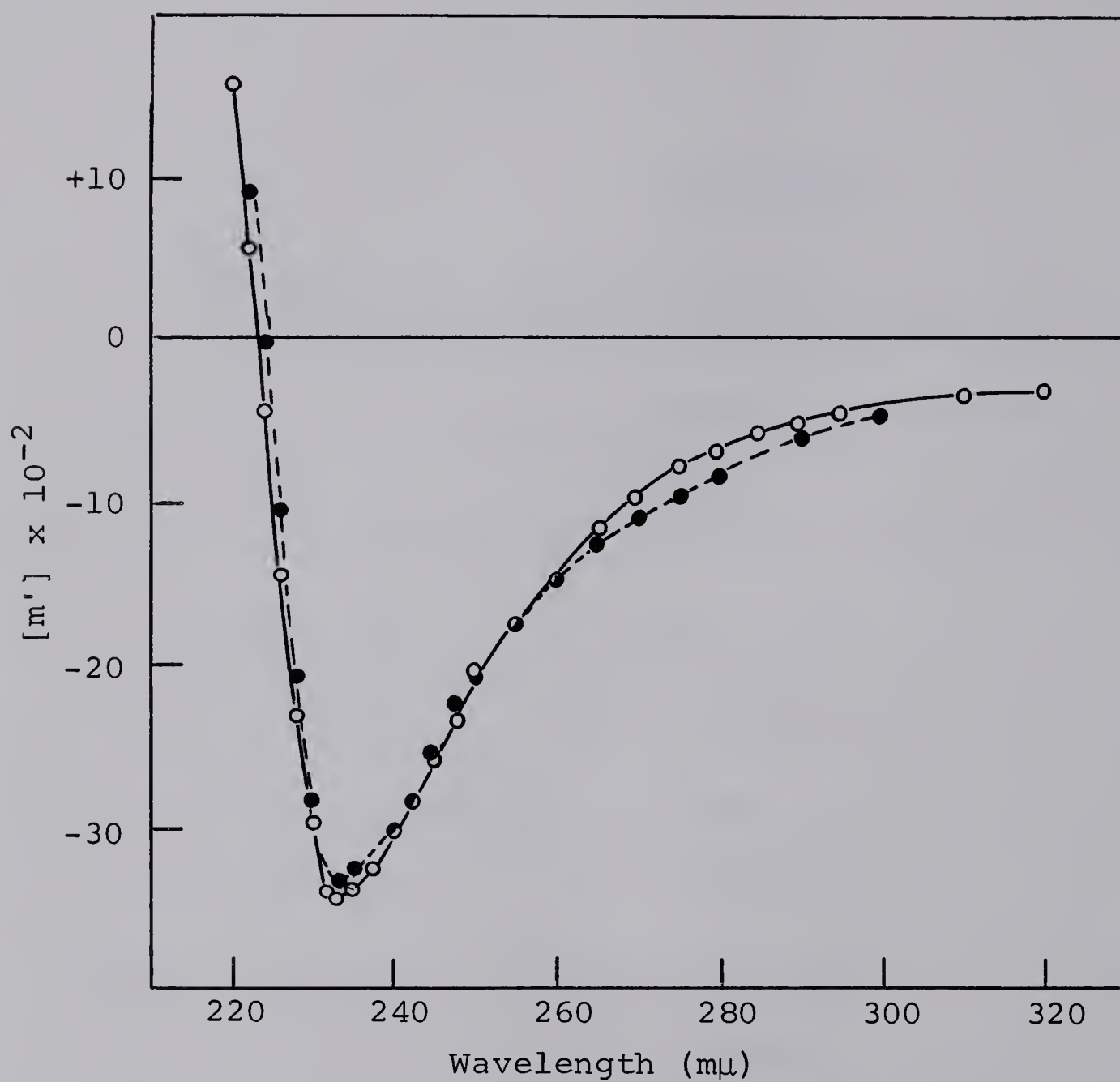


Figure 28. Optical rotatory dispersion of CHTG-B and CHT-B in the ultraviolet region of the spectrum.

—○— CHT-B
 ---●--- CHTG-B

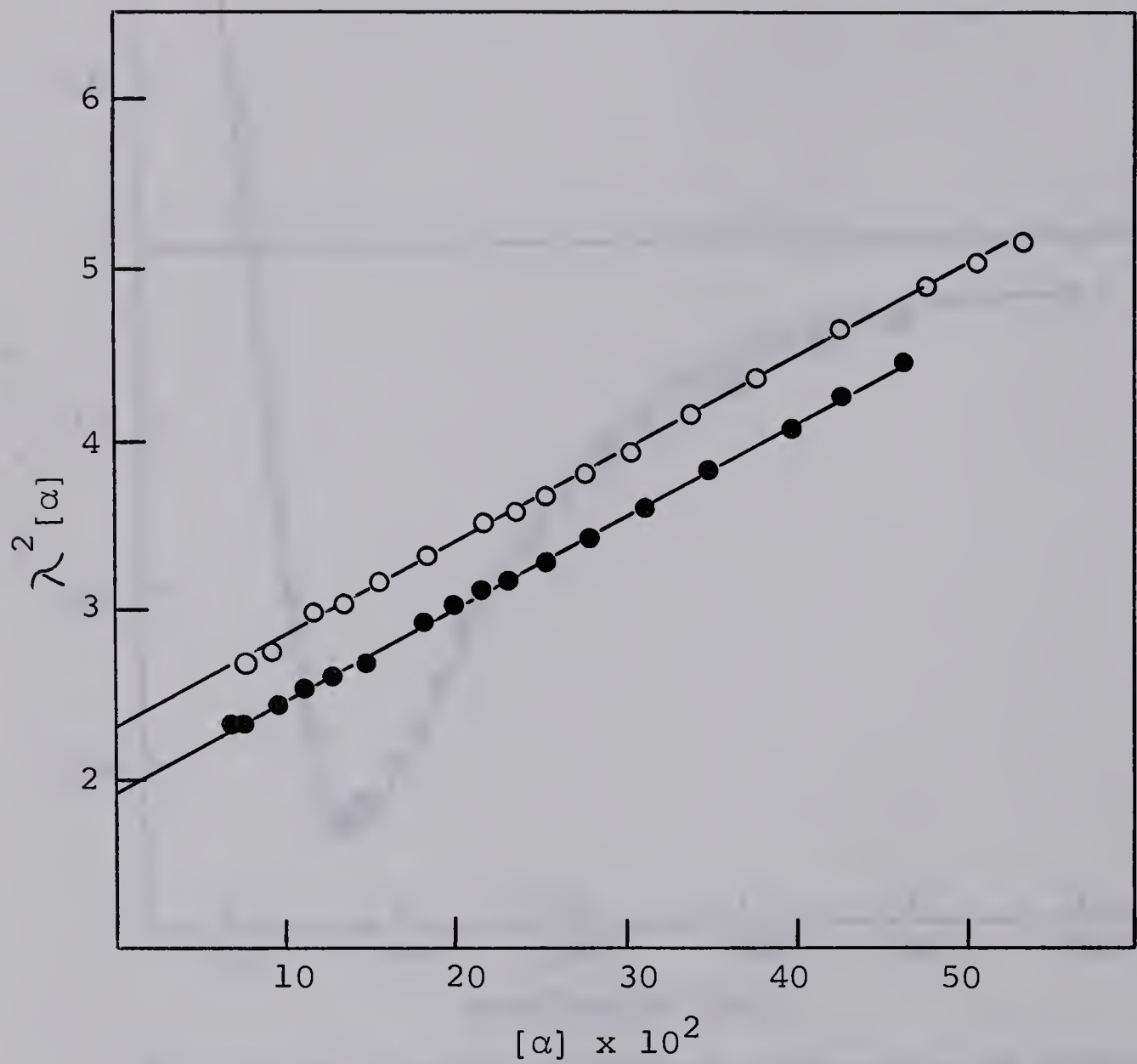


Figure 27. Doty-Yang plot of CHTG-B and CHT-B at pH 3.2.

- CHT-B
- CHTG-B

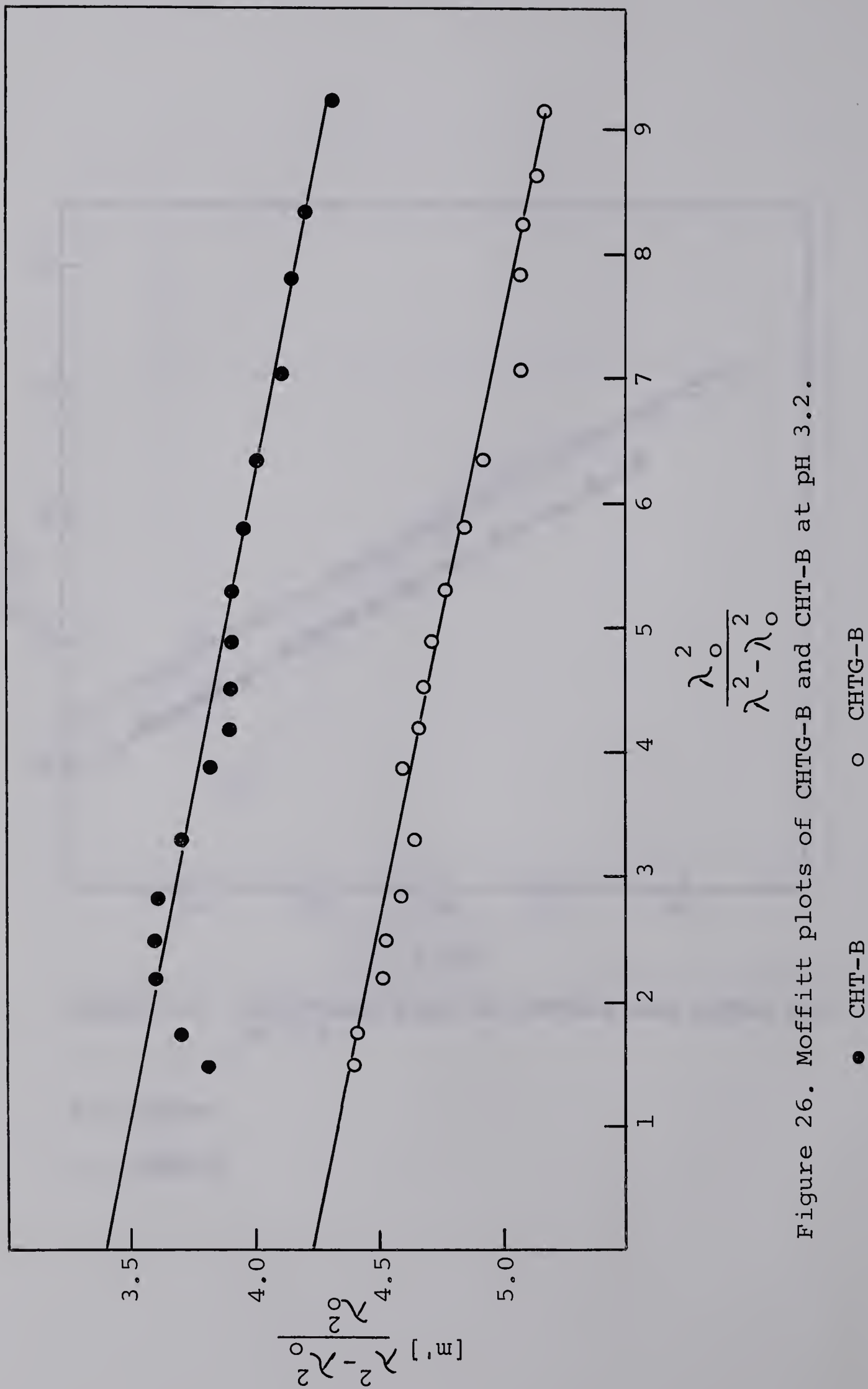


Figure 26. Moffitt plots of CHTG-B and CHT-B at pH 3.2.

to 589 m μ . The angle of symmetry was 2° and the light source was an Osram xenon lamp. Protein concentration was found from measurements of A₂₈₀ m μ .

The observed rotations were analysed to find the helical content of the protein according to the Drude and Moffitt equations.

(ii) Ultraviolet Region

As the observed rotations in this wavelength range are much higher than in the visible region, a solution of 0.03% protein in the same solvent, (0.1 M KCl, 10⁻³ M HCl, pH 3.2) was used). Shorter cells, light path 0.1 and 0.01 dm were filled with protein solution and the optical rotation measured from 330 m μ to 220 m μ . The protein concentration was measured as before and the α observed converted to the reduced mean residue rotation [m'], using a mean residue weight of 106 and $n = 1.365$.

b. Results and Discussion

The results illustrated in Figures 26, 27, 28 and summarized in Table XVI, show that both CHTG-B and CHT-B contain a small proportion of α -helix, involving no more than 14% of the total polypeptide chain. Furthermore, the three analytical methods used all indicate that there is no change in the helical content during activation. The only apparent difference between the two proteins extends over the whole visible range. The zymogen is considerably more laevorotatory than CHT-B, as may be seen in the Moffitt plots, resulting in a difference in a_0 . This change in a_0 may be taken as an

indication of an alteration in the tertiary structure of the zymogen during its conversion to the active enzyme. However, it was observed during the ultracentrifuge experiments that the active enzyme might exist partly in a dimeric form at the concentration and pH at which a_0 was measured. Herskovits et al (117) have recently shown that tetramerization of β -lactoglobulin can result in a_0 becoming less negative while b_0 remains unaffected. The same explanation might be applied to the CHT-B system. Without further measurements of the optical rotatory dispersion under conditions where it is known to be monomeric either explanation for the change of a_0 must remain acceptable.

In the region of the Cotton effect, both zymogen and enzyme show almost classical dispersion curves, each with a sharp trough at 233 $m\mu$ (Figure 28). The depth of the trough is the same for both proteins, indicating, in agreement with the analyses of the optical rotatory dispersion in the visible region, that the percent helix does not change. However, there is an indication that the optical rotation in the region of 280 $m\mu$ is different for the zymogen and the enzyme. The major chromophores at this wavelength are the aromatic side chains of tyrosine and tryptophane which, although they have a high absorbance, possess minor rotational properties and make little contribution to $[m']_{233}$. If, however, as has been demonstrated in Part 3, there is a change in the absorption of one or more of these chromophores during activation, a change in the optical rotatory power may result. The variation in a_0 previously noted indicates that a change in tertiary structure may take place and this change in $[m']$ in the region of 280 $m\mu$ may well

be another manifestation of the same phenomenon. Fasman (personal communication) has noticed a similar perturbation of the optical rotatory dispersion curve of CHT-A in this region.

The low content of α -helix in CHTG-B and CHTG-A and their respective enzymes as determined by optical rotatory dispersion is consistent with what is presently known of the three-dimensional structure of CHTG-A from x-ray diffraction studies. Kraut et al (112) have reported that no recognizable helical sequences are visible from construction of electron density maps of the structure at a resolution of 4 Å. With this protein, there are no rod-like structures straight or long enough to be more than two or three turns of helix. Clearly the α -helix plays only a minor role in the structures of these proteins.

5. Summary

Although no definitive value for the molecular weight of monomeric CHT-B has been obtained in the present work, at low protein concentrations the $S_{20,w}^O$ approaches the $S_{20,w}^O$ value of CHTG-B (2.58) indicating that the two proteins are similar in this respect. This, coupled with the absence of molar amounts of liberated peptides during activation, preclude any significant difference in the molecular weights of the two proteins. However, CHT-B, at pH 3.0 appears to exist in a monomer-dimer equilibrium while the zymogen is monomeric under these conditions.

The isoelectric points of CHTG-B and CHT-B have been shown to be 5.2 and 5.05, respectively. This is in contrast

to the CHTG-A system, where the liberation of the dipeptide, Ser-Arg, causes a large drop in IEP, and is in agreement with the chemical analyses of CHTG-B activation which indicate that no such basic dipeptide is produced.

Conformation changes in the CHTG-B molecule during activation have been investigated by ultraviolet difference spectrophotometry and optical rotatory dispersion. The former indicates that the environment of at least one tyrosine residue is modified during the activation process. Optical rotation measurements in both the visible and ultraviolet regions indicate that no change in the helical content of the zymogen occurs. The observed difference in the Moffitt parameter a_0 between the zymogen and enzyme may be indicative of a change in conformation involving the non-helical or tertiary structure of the molecule. Thus the physical changes accompanying the activation of CHTG-B are very similar to those which take place in the CHTG-A system.

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Appendix A

To show that the chymotryptic inhibitor, indole, has no effect upon carboxypeptidase-A

a. Hydrolysis of the synthetic substrate Cl-acetyl-L-tyrosine

Method

Substrate was prepared in the absence and presence of indole as described below:

	<u>Test</u>	<u>Control</u>
Cl-acetyl-L-tyrosine	0.02 <u>M</u>	0.02 <u>M</u>
KCl	0.1 <u>M</u>	0.1 <u>M</u>
Tris buffer, pH 8.0	0.01 <u>M</u>	0.01 <u>M</u>
indole	0.005 <u>M</u>	-

Carboxypeptidase-A (CP-A) was dissolved in 10% LiCl, forming a 0.1% solution and 50 μ l of this enzyme solution added to 2.0 ml of substrate solution for assay. Samples (100 μ l) were removed at time intervals and added to 2.5 ml of ninhydrin reagent. Colour was developed by heating at 100° for 20 minutes, the tubes were cooled and the coloured solution diluted to 10 ml with 50% v/v ethanol before reading the absorbance at 570 m μ .

Results

	$\Delta A_{570\text{m}\mu}$	
<u>Incubation time</u>	<u>Control</u>	<u>Test</u>
10 minutes	0.317	0.325
20 minutes	0.586	0.573

b. C-terminal analysis of soybean trypsin inhibitor (STI)

Method

Two 25 mg samples of STI (Worthington Biochemical
-110-

Corp.) were each dissolved in 2.0 ml of 0.1 M phosphate buffer, pH 8.0 (0.5 μ mole protein per ml). One sample of buffer was also 0.008 M in indole. Blank samples, 0.5 ml of solution, were removed from each and added to 100 mg Nalcite HCR resin, H^+ form, 15-30 mesh.

To begin digestion 0.3 ml of a 1% solution of CP-A in 10% LiCl was added to each substrate solution (molar ratio of enzyme:substrate = 1:8.5). At time intervals 0.5 ml samples of digestion mixture were treated with 100 mg Nalcite resin. CP-A blanks were also run in the presence and absence of indole. The free amino acids were extracted from the resin as described in Chapter IV.

Aliquots of amino acid solution were separated on Whatman No. 3 chromatography paper by descending chromatography using the solvent system: n-butanol; n-butylacetate; acetic acid; water (19:1:5:25). The upper (organic) phase was used to develop the chromatograms and the lower (aqueous) phase for equilibration. After development, the chromatograms were sprayed lightly with ninhydrin detection spray (50 mg ninhydrin, 25 ml 2 N acetic acid, 75 ml ethanol) to locate the spots. The coloured areas, together with appropriate blanks, were cut out and assayed quantitatively with ninhydrin.

Results

<u>Sample</u>	moles leucine per mole STI (digestion time)		
	<u>2 hrs.</u>	<u>4 hrs.</u>	<u>10 hrs.</u>
Test	0.29	0.33	0.44
Control	0.35	0.33	0.34

Appendix B

The composition of ninhydrin reagent

Acetate buffer, 4M, was made up by dissolving 320 g of NaOH in 1.1 liters of cold water and slowly adding to the stirred solution 670 ml of glacial acetic acid. The final pH was checked and adjusted to pH 5.51 before diluting to 2 liters.

Ninhydrin (1.52 g) was dissolved in 100 ml of acetate buffer, 228 ml of methyl-cellulose added, and the volume made up to 400 ml with water. This solution was brought to 5°, evacuated for 30 minutes, and dry nitrogen gas bubbled through for a further 30 minutes. Hydrindantin (0.34 g) was then added and more N₂ bubbled through. The reagent, stored under N₂ at 5°, was stable for three weeks.

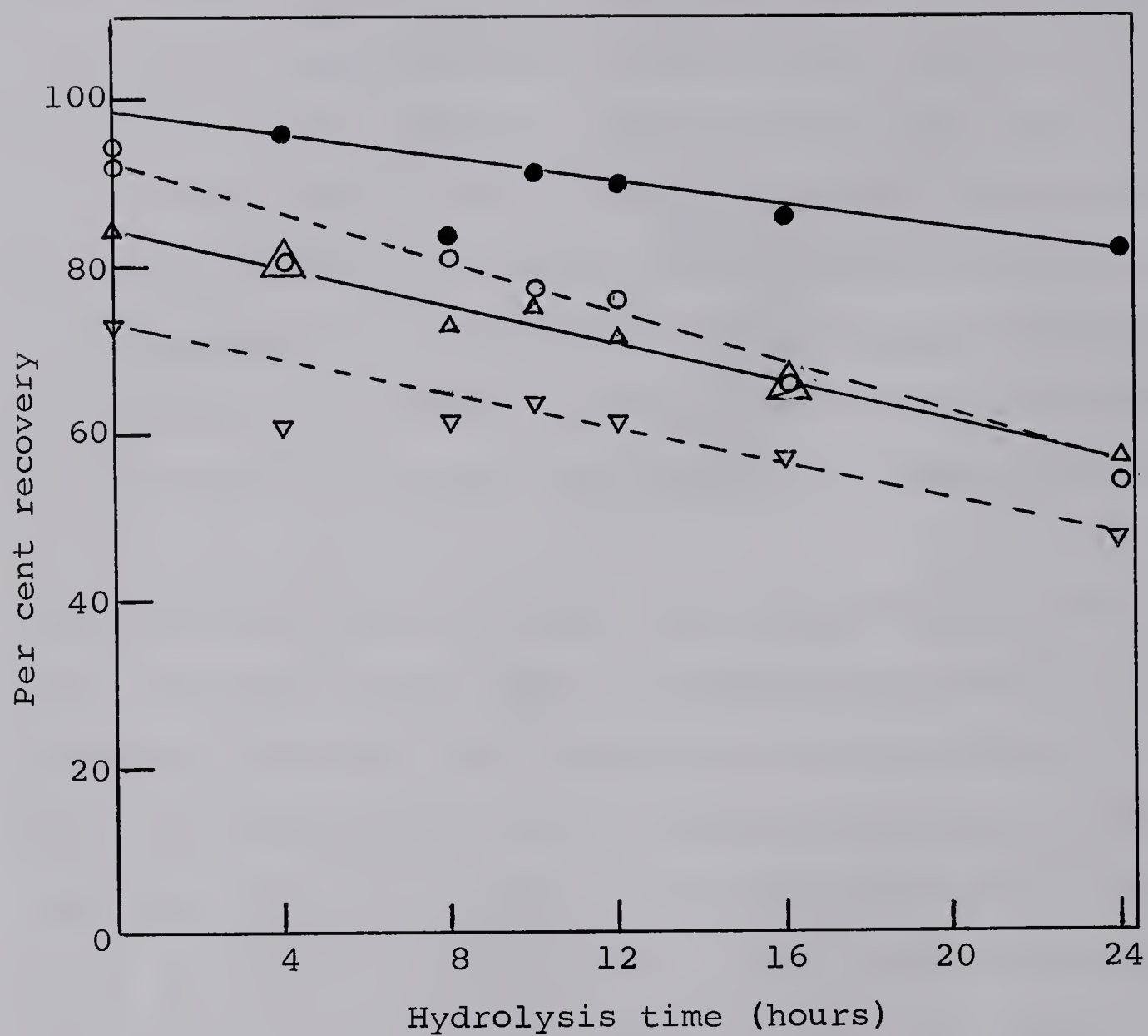
Small volumes of test solution (100-500 µl) were mixed with 1.0 ml of ninhydrin reagent and heated to 100° for 20 minutes. After cooling, the volume was made up to 3.0 ml with 50% v/v aqueous ethanol and the colour density measured at 570 mµ.

Appendix C

To find the losses of DNP-amino acids during hydrolysis ether extraction and chromatography

Solution of DNP-amino acids (Mann Research Labs.) were prepared in acetone and stored for short periods in the dark at 5° in tubes with standard taper stoppers. Exact concentrations were found from the A_{360} of a known volume diluted in 1% sodium bicarbonate and the extinction coefficients (taken from Fraenkel-Conrat et al., 75). Recoveries from chromatography were obtained by separating standard mixtures on Whatman No. 4 chromatography paper and eluting the spots as described in Chapter IV.

Losses of DNP-amino acids may also occur during ether extraction of the acid hydrolysate and during hydrolysis itself. The recovery from these steps was measured by preparing solutions of standard DNP-amino acids, approximately 0.1 μ mole each, and 3 mg of DNP-CHTG-B (from which no DNP-end-group can be extracted by ether) in 0.5 ml of formic acid and 0.5 ml of 6 N HCl. Zero time samples, which indicate the efficiency of ether extraction, were kept at 5° until required. The remainder were heated in sealed, evacuated, pyrex tubes at 105° for the desired time. All tubes were then opened, the contents diluted with 5 ml of water and the DNP-amino acids extracted with four 5 ml volumes of peroxide-free ether. Separation of the mixtures by paper chromatography and quantitative estimation were performed as described in Chapter IV. The results are given in the following table and graph.



Appendix C. The rate of destruction of standard DNP-amino acids during hydrolysis at 105°C . in 6 N HCl-Formic acid.

- DNP-isoleucine
- ▽ bis-DNP-lysine
- DNP-aspartic acid
- △ DNP-alanine

Recovery of DNP-amino acids after ether extraction
and chromatography

Amino acid	bis-DNP-lysine	DNP-iso-leucine	DNP-alanine	DNP-aspartic acid
Molar extinction coeff. at 360m μ (x 10 ⁻⁴)	2.98*	1.78	1.72	1.82
Recovery from chromatography	86	96	96	98
Recovery from chromatography and extraction	72	94	84	92

* λ_{max} for bis-DNP-lysine is 355 m μ at which wavelength $\epsilon = 3.05 \times 10^4$.

Appendix D

A comparison of various dinitrophenylation conditions

Protein	dinitrophenylation conditions	End group	% yield*
CHT-B	pH 10.0, 0.1M KCl, 40°	Ileu	43
act. 1 hour	3 hrs.	Lys	28
CHT-A ₄	pH 8.0, 8M urea, 40% DMF,	Ileu	93
	25°, 2 hrs.	Ala	88
		Lys	17
RNase	pH 8.0, 0.1M KCl, 37°,	Lys	97
	4 hrs.		
RNase	pH 8.0, 0.1M Tris, 50% DMF,	Lys	99
	37°, 5 min.		
RNase	0.1N NaOH, 50% DMF, 25°,	Lys	87
	2 min.		

*All recoveries are corrected for losses during hydrolysis.

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